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**(54) Title:** RECOMBINANT  $\beta$ -1,4 GLUCAN SYNTHASE PROTEINS

**(57) Abstract**

The present invention relates to the compositions and methods associated with the cloning of the subunits of  $\beta$ -1,4 glucan synthases responsible for catalyzing  $\beta$ -1,4 glucan polymer biosynthesis. The invention relates further to compositions and methods for obtaining host cells containing recombinant  $\beta$ -1,4 glucan synthases as well as compositions and methods for obtaining  $\beta$ -1,4 glucan synthase from natural sources. In certain aspects, the present invention provides methods for the cloning of both the 83 Kd subunit and the 93 Kd subunit of the cellulose synthase enzyme from *Acetobacter xylinum*.

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RECOMBINANT  $\beta$ -1,4 GLUCAN SYNTHASE PROTEINS

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This invention relates generally to compositions of and methods for obtaining  $\beta$ -1,4 glucan synthase. The invention relates as well to the DNA sequences encoding  $\beta$ -1,4 glucan synthase, the recombinant vectors carrying those sequences, the recombinant host cells including either the sequences or vectors and recombinant  $\beta$ -1,4 glucan synthase polypeptides. More specifically, the invention relates to the cloning and expression of  $\beta$ -1,4 glucan synthase from Acetobacter xylinum and cotton.

$\beta$ -1,4 glucan biosynthesis such as cellulose biosynthesis is an event largely associated with plant cells, especially certain agronomic species such as cotton where as much as 90-95% of the secondary wall of mature cotton fibers is composed of cellulose. However, studies of  $\beta$ -1,4 glucan biosynthesis in higher plants have been very frustrating, mainly because  $\beta$ -1,4 glucan synthase, due to its lability, has evaded isolation and purification. Furthermore, the study of this enzyme has been hampered by the great difficulty in detecting formation of  $\beta$ -1,4 glucans such as cellulose in vitro using purified preparations of plant and other eukaryotic

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cells [Delmer 1987: In higher plant preparations, the  $\beta$ -1,3-glucan (callose) chains are synthesized instead of the  $\beta$ -1,4-glucan of cellulose]. Frost et al. (1990) have identified a 57 Kd band which was partially purified by 5 product entrapment and is believed to represent the

Apart from the higher plants, a large number of bacteria synthesize  $\beta$ -1,4 glucans such as cellulose, 10 including Acetobacter xylinum (A. xylinum), which converts as much as 35% of the glucose supplied to cultures of this bacterium into cellulose. Unlike the situation in plants and other eukaryotic cells noted above, the cellulose synthase of this bacterium has been isolated to a certain 15 degree. Because of these facts, coupled with the capacity of the bacterium to synthesize a highly purified form of cellulose in vitro using membrane preparations, A. xylinum has become a preferred system for studies on the synthesis and organization of cellulose [Delmer 1987: In the 20 bacterium,  $\beta$ -1,4-glucan (cellulose) chains can be detected].

The biosynthesis of cellulose in A. xylinum is visualized as a two-step process. The first step involves 25 the polymerization of sugar nucleotides (UDP-glucose; UDP-glc) into a  $\beta$ -1,4,-linked glucan chain. The polymerization reaction is catalyzed by the enzyme cellulose synthase (UDP-glucose:1,4- $\beta$ -D-glucosyltransferase; E.C. 2.4.1.12) which is present in the cytoplasmic membrane (Bureau and Brown 1987). The 30 activity of this enzyme is regulated by bis-(3',5')-cyclic diguanylic acid (Ross 1987).

Purification of the cellulose synthase activity from 35 membrane preparations has been accomplished by an entrapment procedure (Lin and Brown 1989) similar in some

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aspects to one used in the purification of chitin synthase from yeast (Kang 1984). The molecular weight of the native enzyme in Triton X-100 solubilized preparations appears to be 490 Kd as determined by gel filtration (Lin and Brown 1989). Electron microscopy of the purified preparation shows doughnut-shaped particles indicating that the cellulose synthase may be organized as a tetramer or octamer (Lin and Brown 1989). Lithium dodecyl sulfate polyacrylamide gel electrophoresis of the purified preparation shows two major bands with molecular weights of 93 Kd and 83 Kd. The resistance of the cellulose synthase activity and of the 83 Kd polypeptide to trypsin treatment has suggested that the 83 Kd polypeptide is the active cellulose synthase (Lin and Brown 1989). The occurrence of these two polypeptides is always observed in purified cellulose synthase preparations of this bacterium, suggesting that they may be subunits of an oligomeric cellulose synthase complex.

Photoaffinity probes have been used with glucan synthases to identify these enzymes in a variety of different species, and with varying subunit molecular sizes. In red beets, a 57 Kd polypeptide has been shown to be the substrate-binding component of (1,3)- $\beta$ -glucan synthase (Wasserman 1989). In cotton fibers and in mung bean, a 50 Kd polypeptide appears to bind the substrate (Delmer and Solomon 1989). In A. xylinum, Mayer et al. (1989) suggested a 67 Kd polypeptide as the substrate-binding subunit and a 57 Kd polypeptide as the activator-binding subunit of a 420 Kd oligomeric cellulose synthase. These investigators suggested the presence of similarly sized peptides in other cellulose-producing organisms such as Agrobacterium tumefaciens, mung bean, wheat, pea and cotton on the basis of immunochemical analyses.

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Genes involved in the synthesis of other exopolysaccharides in a number of bacteria have been cloned using the standard approach of genetic complementation [Easson (1987); Harding (1987); Calvin and 5 Hanawalt (1988)]. Mutants of *A. xylinum* defective in the production of cellulose synthase have been identified in particular strains. Certain of these mutants have been shown to be deficient in the activity of UDPG- 10 pyrophosphorylase, the enzyme required for the synthesis of the cellulose synthase substrate molecule, UDP-glucose. Complementation of these mutants by cloned fragments from *A. xylinum*-derived DNA has led to the isolation of the gene encoding UDPG-pyrophosphorylase (Valla 1989).

15

Recently, a purported operon for bacterial cellulose synthase was described in *A. xylinum* [Wong et al. 1990]. This operon has been suggested to consist of four genes in the order *bcsA*, *bcsB*, *bcsC*, and *bcsD* transcribed as a 20 polycistronic mRNA. The second gene in the operon coding for polypeptide *bcsB* was suggested by these authors to be the cellulose synthase catalytic subunit gene, based on amino acid sequence of a 91 Kd polypeptide purified by product entrapment from trypsin treated membranes and 25 supported by complementation analysis of mutants.

30

Surprisingly, however, no mutants actually deficient in cellulose synthase activity have been identified. This is despite the fact that there have been a large number of mutants identified which morphologically appear to be cellulose-deficient, but which synthesize small amounts of an altered crystalline polymorph of cellulose (cellulose II) and have the wild type level of cellulose synthase activity when assayed *in vitro* for cellulose synthesis 35 [Saxena and Brown (1989); Roberts et al. (1989)].

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Thus, it has been considerably difficult to apply the standard approaches of classical genetics or even the powerful techniques of molecular biology to the study of cellulose biosynthesis. Lacking purified quantities of the enzyme, lacking detailed information of the subunit architecture, lacking means to specifically identify the substrate-binding subunit, and lacking a simple genetic method of isolating mutants deficient in cellulose synthase activity, have each hampered the ability of researchers to isolate DNA segments encoding cellulose synthase. This is particularly true for the higher plants such as the important cellulose fiber-producing cotton species.

The present invention for the first time, provides the vectors, DNA segments, purified protein, antibodies, methods of cloning, and recombinant host cells, seeds and plants necessary to obtain and use a recombinant  $\beta$ -1,4 glucan synthase and related biological molecules. Thus, the difficulties encountered with applying the standard approaches of classical genetics or techniques of molecular biology to the study of cellulose biosynthesis have been overcome. Accordingly, the present invention concerns generally compositions and methods for the preparation of recombinant  $\beta$ -1,4 glucan synthase and related biological molecules of both prokaryotic and eukaryotic origin. Importantly, it is believed that the methods and compositions of matter of the invention may be applied to fiber-producing, agronomically important higher plants such as cotton as well as woody producing plants.

In certain general and overall embodiments, the invention concerns recombinant vectors and isolated DNA segments encoding a  $\beta$ -1,4 glucan synthase peptide or of a  $\beta$ -1,4 glucan synthase-related peptide. The DNA segments of the invention may encode biologically functional

equivalent protein or peptides which have variant amino acid sequences, such as with changes selected based on considerations such as the relative hydropathic score of the amino acids being exchanged.

5

to the class of enzymes capable of polymerizing exopolysaccharides such as the enzyme cellulose synthase which is capable of polymerizing the exopolysaccharide cellulose. It is understood that in referring to a  $\beta$ -1,4 glucan synthase, the present application refers to holoenzyme which may, in some cases, include a multiple subunit molecule. In certain instances, the holoenzyme may be composed of multiple copies of the same polypeptide. In other cases, the holoenzyme may be composed of single or multiple copies of different polypeptides. For instance, in the case of the glucan synthase, cellulose synthase, the native holoenzyme when solubilized from membranes of A. xylinum appears to be a multiple subunit with a molecular weight of 490 Kd. These holoenzymes appear to be composed of multiple copies of at least two different polypeptides of 83 and 93 Kd molecular weights organized into tetramers or octamers. Therefore, when referring to the  $\beta$ -1,4 glucan synthase, cellulose synthase, it is understood to include both polypeptides. In some instances, the terms "glucan synthase" or "glucan synthase-related" are used inter-changeably with " $\beta$ -1,4 glucan synthase" or " $\beta$ -1,4 glucan synthase-related."

30

Where it is possible to distinguish the function of a given polypeptide, the function is identified by the modifier "catalytic" where a catalytic function can be ascribed to the polypeptide. Where catalytic function has not yet been ascribed to the polypeptide, but such peptide is known to be associated with glucan biosynthesis, the modifier "glucan synthase-related" is used.

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More specifically, in the context of the present invention, the term  $\beta$ -1,4 glucan synthase is intended to refer to peptides or proteins having the biological and the immunological identity of the cellulose synthase of the cell lines enabled by the present invention. For example, such cell lines would include cells of Acetobacter xylinum. Generally, the A. xylinum cellulose synthase of the invention will refer to a 723 amino acid peptide or protein in that this is the precise length of the only presently sequenced cellulose synthase. However, the invention does not preclude and, in fact enables, preparation or use of shorter or longer peptides or proteins, so long as a peptide or protein has similar in kind biological activity and/or a cross reactive immunological reactivity, for example, as defined by rabbit polyclonal antisera. For instance, the other wild type strain of A. xylinum ATCC 23769 which was used by the present inventors possesses a 75 Kd polypeptide as a catalytic subunit of cellulose synthase by using the methods of the invention. Similarly, certain of the glucan synthase proteins of the cotton cells described herein may represent analogous proteins in the cotton fiber cell to the 83 Kd cellulose synthase of A. xylinum.

The term "cellulose synthase-related" is intended to refer to both DNA sequences, and peptides or proteins having the biological and the immunological identity of the cellulose synthase-related proteins of the cell lines enabled by the present invention. Therefore, the cellulose synthase-related proteins of the present invention would include the cellulose synthase catalytic subunit as well as any protein which co-purifies with the catalytic subunit in a tightly associated complex. In A. xylinum, such cellulose synthase-related proteins would include the 723 amino acid catalytic subunit as well as the tightly associated 809 amino acid polypeptide.

In addition, "cellulose synthase-related" may be used in conjunction with DNA sequences. Therefore, the cellulose synthase-related DNA sequences of the present invention would include DNA sequences encoding the 5 cellulose synthase catalytic subunit as well as the DNA sequences encoding

catalytic subunit in a tightly associated complex.

Furthermore, once such DNA sequences are identified, the present invention enables one to identify further 10 cellulose synthase-related proteins which are found to be contained in an operon, or other coordinately controlled genetic system, with the catalytic cellulose synthase protein.

15 In certain general aspects, the invention relates to the preparation and use of DNA segments, including vectors or DNA fragments, having a sequence encoding a  $\beta$ -1,4 glucan synthase polypeptide or related polypeptides. For vectors, any number are known in which DNA sequences of 20 the invention may be incorporated. The vector pUC18 has been demonstrated to be of particular value. Likewise, the related vectors M13mp18 and M13mp19 may be used in certain embodiments of the invention, in particular, in performing dideoxy sequencing.

25

In certain embodiments, the vector will contain a substantially purified DNA fragment which encodes at least a useful portion of a cellulose synthase polypeptide which 30 includes the amino acids 1 to 723 of Figure 1, or functionally equivalent amino acids. Alternatively, the vector will contain a substantially purified DNA fragment which encodes at least a useful portion of a cellulose synthase-related polypeptide which includes the amino 35 acids 1 to 809 of Figure 13, or functionally equivalent amino acids. Recombinant vectors and isolated segments

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may, therefore, variously include the basic  $\beta$ -1,4 glucan synthase or related molecule coding region itself or may contain coding regions bearing selected alterations or modifications in the basic coding region of  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related molecules.  
5 Alternatively, such vectors or fragments may encode larger proteins or peptides which nevertheless include the basic coding region or regions. The vectors or fragments may encode both the glucan synthase and glucan synthase-related proteins, or parts of such combinations. The vectors and fragments which encode combinations of glucan synthase and glucan synthase-related proteins may contain such combinations as they are organized in naturally occurring gene sequences (such as in operons) or such  
10 combinations may be organized differently from those encountered in naturally occurring gene sequences. In any event, it should be appreciated that due to codon redundancy, as well as biological functional equivalence, this aspect of the invention is not limited to the  
15 particular DNA sequences shown in Figures 1 or 13.  
20

Recombinant vectors such as the foregoing are useful both as a means for preparing quantities of the  $\beta$ -1,4 glucan synthase-encoding DNA or  $\beta$ -1,4 glucan synthase-related protein-encoding DNAs themselves, and as a means  
25 for preparing the encoded protein and peptides. It is contemplated that where glucan synthase or glucan synthase-related proteins of the invention are made by recombinant means, one may employ either prokaryotic or  
30 eukaryotic expression and shuttle systems.

Prokaryotic host cells are disclosed in a preferred embodiment of the invention. However, in that prokaryotic systems are usually incapable of correctly processing eukaryotic precursor proteins, and since eukaryotic  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related proteins  
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are anticipated using the teachings of the disclosed invention, one may desire to express such sequences in eukaryotic hosts. However, even where the DNA segment encodes a eukaryotic glucan synthase or glucan synthase-related protein, it is contemplated that prokaryotic

10 therefore, the invention can be used in combination with vectors which can shuttle between the eukaryotic and prokaryotic cells. Such a system is that of the Ti plasmids used in conjunction with the bacteria Agrobacterium tumefaciens.

15 Where expression of  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein in a eukaryotic host is contemplated, it most likely will be desirable to employ a vector such as a plasmid, that incorporates a eukaryotic origin of replication, such as those of the CaMV (cauliflower mosaic virus) and plasmids derived therefrom. Additionally, for the purposes of expression in eukaryotic 20 systems, one will desire to position the glucan synthase or glucan synthase-related protein encoding sequence adjacent to and under the control of an effective eukaryotic promoter such as promoters used in combination with Ti plasmids. To bring a coding sequence under 25 control of a promoter, whether it is eukaryotic or prokaryotic, what is generally needed is to position the 5' end of the translation initiation site of the proper translational reading frame of the protein between about 1 and about 50 nucleotides 3' of or "downstream" with 30 respect to the promoter chosen. Furthermore, where eukaryotic expression is anticipated, one will typically desire to incorporate into the transcriptional unit which includes the glucan synthase or glucan synthase-related protein, an appropriate polyadenylation site. Typically, 35 the polyadenylation site is placed about 30-2000

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nucleotides "downstream" of the termination site of the protein at a position prior to transcription termination.

Accordingly, in certain preferred embodiments, the vectors of the invention are those where the  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein polypeptide encoding sequence is positioned adjacent to and under the control of an effective promoter. The vectors may be that set of vectors known well to those of skill in the art where the promoter comprises a prokaryotic promoter, the vector being adapted for expression in a prokaryotic host. Alternatively, the vectors may be those of common knowledge to skilled artisans where the promoter comprises a eukaryotic promoter, and the vector further includes a polyadenylation signal positioned 3' of the carboxy-terminal amino acid, and within a transcriptional unit of the encoded protein.

In certain embodiments of the invention, it is contemplated that DNA fragments both shorter and longer which incorporate sequences from Figure 1 or Figure 13 will find additional utilities, including uses as short DNA fragment hybridization probes, e.g., in screening both prokaryotic and eukaryotic recombinant clone banks. In any event, fragments including the sequence in Figure 1 or Figure 13 for stretches as short as 14 or so nucleotides, will generally find utility in accordance with these or other embodiments. By having stretches of at least about 14 nucleotides in common with the glucan synthase DNA sequence of Figure 1 or of the glucan synthase-related DNA sequence of Figure 13, or their complements, a DNA segment will typically have the ability to form a preferential hybridization with glucan synthase species DNA, particularly under more stringent conditions such as 0.15 M sodium chloride and 0.02 M sodium citrate, pH 7.4 at

about 50°C. While such a complementary or common stretch will typically ensure the ability to form a stable hybrid, longer stretches of complementary DNA may prove more desirable for certain embodiments. Thus, one may desire to use DNA segments incorporating longer stretches of complementary DNA, even 25 or so bases.

The invention also provides methods for isolating glucan synthase or cellulose synthase-related polypeptides from both recombinant and non-recombinant sources. Such a protein will typically include an amino acid sequence including to amino acids 1 to 723 of Figure 1 or including to amino acids 1 to 809 of Figure 13, respectively. However, the methods of the invention have been demonstrated to be successful in isolating a 75 Kd cellulose synthase polypeptide from *A. xylinum* strain ATCC 23769 as well as glucan synthase polypeptides of 66 Kd, 57 Kd and 35 Kd from cotton fiber cells. It will be apparent for instance, to those of skill in the art in light of the present specification that the 75 Kd polypeptide is different in molecular weight from the 83 Kd polypeptide described extensively herein. Furthermore, it will be obvious to such skilled artisans that the differences in molecular weights of these two polypeptides may result from actual differences in the primary structure of the amino acid chains themselves or may result from any number of post-translational modifications. Since such a protein represents the sequence for the catalytic subunit of the cellulose synthase protein, such a protein may be used directly to synthesize cellulose when provided the proper environment and substrate. Additionally, such a protein may be used to prepare an antibody for use in certain embodiments which antibody may be either a polyclonal or a monoclonal antibody and which, in any case, represents an

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antibody immunologically reactive with any of the polypeptides of the invention.

5        The invention provides, therefore, a method of producing a recombinant  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein polypeptide. This method includes the use of a recombinant host cell where the recombinant host cell is capable of expressing a recombinant glucan synthase or glucan synthase-related 10 protein polypeptide. Furthermore, the method for producing recombinant glucan synthase or glucan synthase-related protein provided in the invention includes culturing the host cell under conditions appropriate for expressing the polypeptide. Finally, the method of 15 production claimed would include collecting the polypeptide thus expressed.

20        The method is particularly applicable where one desires to obtain a polypeptide corresponding to an 83 Kd catalytic subunit of cellulose synthase or of a cellulose synthase-related subunit such as the 93 Kd polypeptide of Acetobacter xylinum. However, it is proposed that the method may be directed to isolation of a glucan synthase polypeptide and a gene that is encoding a protein that is 25 substantially similar to the 83 Kd catalytic subunit.

30        In particular embodiments, the method will typically involve selecting cells that are capable of glucan biosynthesis, particularly cells of Acetobacter xylinum and cotton fiber. A variety of cells are amenable to the method of the invention, for instance, cells of Agrobacterium, Rhizobium, Alcaligenes and particularly Sarcina. Of course, methods will typically involve culturing cells such that  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 35 glucan synthase-related protein is produced. In most cases, the cell lines used in the method will be one of

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those cell lines which can actively engage in cellulose biosynthesis without addition of extraneous activators. Where cells of Acetobacter xylinum will be used, cultures will be grown in aerated and agitated culture medium 5 containing Celluclast in order to free the cells trapped in the product cellulose.

..... as an inoculum (see U.S. Patent

Application, Serial No. 022,904, filed March 6, 1987, Brown et al.).

10

In certain embodiments,  $\beta$ -1,4 glucan synthase may be partially purified from cells by solubilizing the cellular membranes. Partially purifying glucan synthase from cells by solubilizing the cellular membranes is preferably 15 accomplished using digitonin. However, other solubilizing agents may be employed as long as the glucan synthase enzyme thus solubilized retains a substantial amount of its native activity. Other solubilizing agents which may be used include n-octyl glucoside and other nonionic 20 detergents or Triton X-100.

Moreover, one may desire to even further purify the enzyme. If so, it has been found that a particularly useful approach employs "product entrapment". Product 25 entrapment is explained in greater detail in the examples which follow. This technique entails generally layering solubilized enzyme and the substrate UDP-Glu solution on top of a cushion, e.g., of buffered glycerol, allowing biosynthesis of the  $\beta$ -1,4 glucan polymer to occur, 30 centrifuging the resulting mixture and recovering the pellet which consists of  $\beta$ -1,4 glucan polymer along with the entrapped enzyme. This may even be followed by a series of chromatographic steps to still further purify the protein.

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After obtaining a partially purified glucan synthase one may desire to admix it with a cellulose synthase activator in order to enhance the activity of the purified cellulose synthase. A useful cellulose synthase activator 5 is bis-(3',5')-cyclic diguanylic acid in A. xylinum. In cotton fiber cells, bis-(2',3')-cyclic adenosine monophosphate is a useful activator. In addition, the present inventors have discovered that crude extracts of 10 A. xylinum function as activators to achieve significant increases in azido-UDP-Glu binding in cotton fiber cells which fail to be enhanced significantly by purified cyclic-di-GMP. Thus, activators may be both well-characterized chemical substances or may represent complex and only roughly characterized substances as long as 15 azido-UDP-Glu binding is enhanced in the target cell.

The present inventors have found a photoincorporation method to function in identifying the catalytic subunit of the  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related 20 protein. Photolabeling may be desirable where other modes of labelling the active enzyme are insufficient. Photoincorporation of glucan synthase or cellulose synthase-related protein is accomplished most readily using a radioactively labeled azidonucleotide sugar analog 25 capable of specifically interacting with the nucleotide sugar binding site of glucan synthase or glucan synthase-related protein. The photoincorporation technique is applicable to vascular plant cells like cotton fiber cells as well as to bacterial cells possessing glucan synthase 30 or glucan synthase-related proteins.

Upon sequencing the purified  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein, one may construct oligonucleotide probes corresponding generally to some 35 portion of the DNA segment which would encode such amino acid sequences as are determined from amino acid

sequencing the purified glucan synthase or glucan synthase-related protein. Oligonucleotide probes which incorporate particular DNA sequences will preferably correspond to at least a portion of the amino acid sequences of the various cellulose synthase or cellulose synthase-related protein polypeptides.

10 Preferably, these probes will be DNA sequences which encode portions of the Acetobacter xylinum 83 Kd cellulose synthase polypeptide or 93 Kd cellulose synthase-related polypeptide. In any case, the resulting probes will be used to probe a suitable source of DNA from a cell line capable of glucan biosynthesis.

15        The probing will usually be accomplished by  
hybridizing the oligonucleotide probes to a DNA source  
suspected of possessing a  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4  
glucan synthase-related protein gene. In some cases, the  
probes will constitute only a single probe, in others, the  
probes will constitute a collection of probes based on a  
certain amino acid sequence of the glucan synthase or  
glucan synthase-related protein and will account in their  
diversity for the redundancy inherent in the genetic code.

25 A suitable source of DNA from a cell line capable of  
glucan synthase or glucan synthase-related protein  
expression may be a genomic library of the cell line of  
interest. Alternatively, the suitable source of DNA may  
include total DNA from the cell line of interest. Once  
30 the hybridization process of the invention has identified  
a candidate DNA segment, one will desire to confirm that a  
positive clone has been obtained, e.g., by further  
hybridization, sequencing and\or expression and testing.

35 The invention also provides a means for obtaining a  
variety of recombinant host cells which incorporate a DNA

sequence in accordance with that depicted in Figure 1 or Figure 13. The host cell may be either prokaryotic or eukaryotic in nature. In any case, it is understood that the DNA segment encoding a glucan synthase or glucan synthase-related polypeptide will also possess the regulatory signals functional in the particular host cell. A preferred embodiment includes a recombinant plant entity which may comprise a plant cell (e.g., tissue cultured plant cells), a recombinant seed, or a recombinant plant having an incorporated gene encoding a glucan synthase or cellulose synthase-related polypeptide. The plant entity will possess the recombinant gene as a result of the in vitro introduction of the gene into a plant cell, wherein the recombinant gene is under the transcriptional control of regulatory signals functional in the particular plant entity. These regulatory signals will be appropriately selected to control the expression of the recombinant glucan synthase or glucan synthase-related protein in a manner to allow all requisite transcriptional and post-transcriptional modification.

Of particular interest in this regard will be plant entities which are cultivated for their content of glucan biopolymers such as cellulose and, most particularly, will be such plant entities as species of the genus Gossypium. Alternatively, more primitive entities such as algae or cyanobacterium are included within the description. Importantly, the gene encoding glucan synthase or glucan synthase-related protein may either be a gene which is heterologous to the plant cell, seed or plant in which the gene is introduced or it may be a copy of the gene homologous to that found in the plant entity being transformed. As used herein, the terms heterologous and homologous refer to the source of the recombinant DNA in reference to the DNA of the host cell. Thus, for instance, A. xylinum DNA from a particular strain of this

bacterium which is encoding a cellulose synthase or cellulose synthase-related polypeptide if used to transform another strain of cellulose synthase or cellulose synthase-related protein-producing bacteria or if used to transform a eukaryotic host cell would be the incorporation into either the

heterologous to that of the host cell's DNA. Conversely, the incorporation of the same A. xylinum DNA as above by retransforming an identical strain of A. xylinum would be the incorporation of a DNA segment homologous to that of the host cell's DNA.

15        In certain general aspects, then, a method of  
producing a glucan synthase or glucan synthase-related  
polypeptide is provided by the invention. First, one  
produces a recombinant host cell according to the methods  
and with the compositions of the invention such that the  
recombinant cell so produced is capable of expressing the  
polypeptide. Next, one cultures the host cell under  
conditions appropriate for expressing the polypeptide.  
20        Finally, according to the methods and with the  
compositions of the invention the recombinant polypeptide  
is recovered.

25        In other general aspects, a method of producing a  $\beta$ -1,4 glucan polymer is provided using the methods and compositions of the invention. Depending upon the particular glucan synthase one selects, one may direct the method to produce a given glucan polymer. For instance,  
30        if one desires a pure  $\beta$ -1,4 glucan polymer, one chooses a  $\beta$ -1,4 glucan synthase of the present invention such as the glucan synthase of Acetobacter. Of course, one may also desire to produce mixed glucan polymers in which case one may select a combination of glucan synthases of the  
35        invention. By way of example, if one desired to use the method to produce a  $\beta$ -1,4 glucan polymer, one first

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obtains one of the recombinant host cells of the invention using the methods and compositions of the invention such that the host cell so obtained is capable of producing the  $\beta$ -1,4 glucan polymer. Next, one cultures the host cell 5 under conditions appropriate for producing the  $\beta$ -1,4 glucan polymer. Finally, one recovers the  $\beta$ -1,4 glucan polymer thus produced.

It is also possible, using the methods and 10 compositions of matter of the present invention to cause certain polymorphs of  $\beta$ -1,4 glucans to be exclusively or almost exclusively produced in vitro. By altering substrate concentrations made available to the enzyme preparations capable of producing the  $\beta$ -1,4 glucan, the 15 present inventors can select, for instance, the nearly exclusive in vitro production of cellulose I polymorph from the enzyme derived from A. xylinum. This cellulose polymorph possesses many desirable characteristics such as the native cellulose I polymorph synthesized by living 20 cells.

It will be obvious to those of skill in the art that the recombinant  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related polypeptide expressed by these host cells 25 may use as its sole substrate UDP-glucose and produce a homopolymer such as cellulose. Alternatively, the substrate for the recombinant polypeptide may be other nucleotide sugars or mixtures of nucleotide sugars with UDP-glucose such as UDP-xylose and will result in 30 heteropolymers. Furthermore, the recombinant glucan synthase or glucan synthase-related protein may act alone in these host cells or may act in concert with other enzymes to produce mixed polymer compositions.

35 Fig. 1. DNA/protein sequences of the A. xylinum cellulose synthase catalytic subunit gene. The coding

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sequence begins at DNA position 636 and ends at DNA position 2804. The protein sequence begins at amino acid position 1 and ends at amino acid position 723. The single letter code used in the protein sequence here corresponds to amino acyl residues as follows: A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; Y, tyrosine; W, tryptophan; V, valine.

Fig. 2. 8% LDS-PAGE of cellulose synthase purified from Acetobacter xylinum. Lanes 1 to 5 were stained with Coomassie blue; lanes 6 to 8 were stained with silver. Lane 1, TS (20  $\mu$ g), Triton X-100-solubilized enzyme preparation; Lane 2, TSt (20  $\mu$ g), Triton X-100-solubilized enzyme preparation from trypsinized washed membranes; lane 3, TS-EP<sub>1</sub> (5  $\mu$ g), the purified enzyme by one step of entrapment, EP<sub>1</sub>, from the TS; lane 4, TSt-EP<sub>1</sub> (5  $\mu$ g), the enzyme purified by one step of entrapment, EP<sub>1</sub>, from TSt; lane 5, TSt-EP<sub>2</sub> (40 $\mu$ g), the enzyme purified by two steps of entrapment, EP<sub>2</sub>, from the TSt. This lane was overloaded deliberately in order to show the purity of the enzyme; lane 6, TSt-EP<sub>1</sub> (3.0  $\mu$ g); lane 7, TSt-EP<sub>2</sub> (p.5  $\mu$ g); lane 8, TSt-EP<sub>2</sub> (1.5  $\mu$ g).

Fig. 3. 8% LDS-PAGE of cellulose synthase in various purified fractions by using two detergents. Lane 1 to lane 4 were stained with Coomassie blue. Lane 1, OGS-EP<sub>1</sub> (10  $\mu$ g), the EP<sub>1</sub> purified from the n-octyl glucoside-solubilized enzyme preparation (OGS); lane 2, OGSt-EP<sub>1</sub> (10  $\mu$ g), the EP<sub>1</sub> purified from OGSt; lane 3, DS-EP<sub>1</sub> (5  $\mu$ g), the EP<sub>1</sub> purified from digitonin-solubilized enzyme; lane 4, DS-EP<sub>1</sub> (10  $\mu$ g), the purified preparation from DS, but without 30% glycerol cushion during the entrapment.

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Fig. 4. Subunit composition of the purified enzyme: effect of reduction and trypsin. (A) 8% LDS-PAGE of the EP<sub>1</sub> fraction from TS prepared from trypsinized washed membranes. Samples (10 µg protein per lane) were 5 electrophoresed in sample buffer with mercaptoethanol (lane 1) and without it (lane 2). Note the prominence of the 93- and 83 Kd bands in lane 2. (B) 8% LDS-PAGE of the EP<sub>1</sub> fraction from DS in the absence of reducing agent. Samples (10 µg protein per lane) were gently shaken at 10 25°C in the presence of trypsin (trypsin/EP<sub>1</sub> = 4 µg/1,000 µg of protein concentration) for two minutes (lane 3), five minutes (lane 4), 10 minutes (lane 5) and 0 minutes (lane 6).

Fig. 5. Elution of cellulose synthase from a Con-A-Sepharose column. A 3 ml sample of the TS fraction was passed through a 7.5-ml Con A-Sepharose column which was then washed with enzyme buffer. The enzyme was eluted with 0.3 M methyl α-D-mannopyranoside in the enzyme buffer 20 (arrow). Fractions of 1 ml were collected.

Fig. 6. Photographs of 8% LDS-PAGE without reducing agent, and stained either by the Schiff-periodate method using dansyl hydrazine (lane 1) or with a FITC-Con A (lane 25 2). Each lane contains 10 µg of the EP<sub>1</sub> purified from DS.

Fig. 7. Gel filtration elution pattern of TS and trypsin-treated TS on Sephadryl S-300. A 3 ml sample was applied. Fractions of 3 ml were collected. (A) elution 30 profile for TS; (b) elution profile for TS which was treated with trypsin (5 µg/ml) at 25°C for 10 minutes.

Fig. 8. Photoaffinity labeling of cellulose synthase in various enzyme samples using 20 µM of [ $\beta$ -<sup>32</sup>P]5N<sub>3</sub>UDP-glc. Samples in lanes 1 and 2 are the membrane fractions, 40 µg 35 of protein per lane. Samples in lanes 3 and 4 are the

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5 digitonin-solubilized enzyme (20  $\mu$ g of protein per lane). Samples in lanes 5 and 6 are the purified cellulose synthase from digitonin-solubilized preparations (3  $\mu$ g of protein per lane). Either plus (+) or minus (-)  $\mu$ l of cellulose synthase activator contained in reaction mixture is indicated.

10 Fig. 9. Coomassie Blue stained 8% gel (A) and its corresponding autoradiography (B) of the cellulose synthase in purified fractions prepared by three detergents, digitonin, Triton X-100, and n-octyl glucoside, respectively. Samples in lanes 1 and 2 are the enzymes purified from the digitonin-extracted preparations (5  $\mu$ g of protein per lane). The sample in lane 3 is the 15 enzyme purified from the n-octyl glucoside-extracted preparations (2.35  $\mu$ g of protein). The sample in lane 4 is the enzyme purified from the Triton X-100-extracted preparations (5  $\mu$ g of protein). The reaction mixture not illuminated with UV light is shown in lane 1.

20 Fig. 10. Autoradiograph demonstrating the prevention of  $[\beta-^{32}P]5N_3$ UDP-glc photoinsertion into the 83 Kd polypeptide by various concentrations of UDP-glc; 3  $\mu$ g of the enzyme purified from digitonin-extracted preparations 25 for each lane. UDP-glc was added to reaction mixture at final concentrations of 0 (lane 1), 50 (lane 2), 100 (lane 3), and 300  $\mu$ M (lane 4).

30 Fig. 11. Autoradiograph demonstrating the saturation of  $[\beta-^{32}P]5N_3$ UDP-glc photoinsertion into the 83 Kd polypeptide. The final molar concentrations of  $[\beta-$  35  $^{32}P]5N_3$ UDP-glc in the reaction mixtures were 20 (lanes 1 and 2), 50 (lane 3), 100 (lane 4), 300 (lane 5), and 500  $\mu$ M (lane 6), respectively. Use of preirradiated  $[\beta-$   $^{32}P]5N_3$ UDP-glc is shown in lane 1.

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Fig. 12. Autoradiograph demonstrating the labeling of a 57 Kd polypeptide in the digitonin-solubilized enzyme preparations. note that contaminating ( $^{32}\text{P}$ )glc-1-P may be present in the preparation of the [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc probe. The digitonin-solubilized enzymes, 20  $\mu\text{g}$  of protein per lane, were incubated with 40  $\mu\text{M}$  of [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc (4 mCi/ $\mu\text{mole}$ ) either alone, lanes 1 to 3, or in the presence of 500  $\mu\text{M}$  glc-6-P (lane 4), glc-1-P (lane 5), and glc-1,6-P (lane 6). For the control experiments, the preirradiated [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc was used (lane 1) or the reaction mixture not irradiated as shown in lane 2.

Fig. 13. DNA sequence and deduced amino acid sequence of the 93 Kd polypeptide of *A. xylinum* cellulose synthase-related protein. The DNA sequence is numbered such that it is in continuation of the sequence in Figure 1. Numbers to the right of each line in the first column are the amino acid positions and in the second column are the nucleotide positions. The sequence coding for the cellulose synthase catalytic subunit stops at nucleotide position 2804 and the sequence for the 93 Kd polypeptide starts from position 2860. A potential ribosome-binding site for the 93 Kd polypeptide is marked by double lines under the sequence. The signal sequence (amino acid positions 1-16) is shown in italics. N-terminal amino acid sequence determined for the 93 Kd polypeptide and found to be matching with the deduced amino acid sequence (positions 27-40) is underlined. The transmembrane segment (amino acid positions 586-606) is indicated by dotted underline.

Fig. 14. Typical example from labeling experiments using azido-UD- $^{32}\text{P}$ -Glu of plasma membrane fractions from cotton fibers. Membranes from a 25 days post- anthesis ovary were used. Lane 1, no UV + activator; Lane 2, no activator + UV; Lane 3, crude GX activator from

Acetobacter; Lane 4, fraction "X-1" from crude GX above; Lane 5, fraction "X-2" from crude GX above; Lane 6, fraction "X-3" from crude GX above; Lane 7, heated supernatant from Acetobacter crude factor; Lane 8, 50 uM DCB; Lane 9, 10 uM pure c-di-GMP; Lane 10, phosphodiesterase treated GX. Lanes 11-15 show the same pattern as lanes 1-5, but with a different set of bands (37, 36, 49, 31, 21, and 14 kDa polypeptides).

10 Fig. 15. Negatively stained preparation of trypsin-  
treated/Triton-solubilized membrane fraction of  
Acetobacter which has been incubated in the presence of  
20mM UDP-glc, showing the cellulose synthase enzyme  
complexes (large arrows) and associated in vitro cellulose  
I product (small arrows).

15            In accordance with the present invention, access is  
provided to both recombinant and non-recombinant  $\beta$ -1,4  
glucan synthase or  $\beta$ -1,4 glucan synthase-related protein.  
Methods are provided herein for the preparation of such  
20          enzymes in a substantially purified state. Since glucan  
polymers are ubiquitous throughout the plant kingdom, and  
since great effort has gone into attempts to isolate these  
highly important plant enzymes, the disclosure of this  
invention constitutes a true watershed in glucan polymer  
25          biochemistry.

The commercial applications of recombinant  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein and recombinantly produced glucan polymer have only begun recently to be explored since up until the present invention, no recombinant gene encoding a catalytic subunit of a glucan synthase or glucan synthase-related protein had been isolated. The present invention, provides such a capability for the first time. In preferred embodiments, the recombinant genes for cellulose synthase or cellulose synthase-related protein represented

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by this invention are obtained using a microbial cell line. However, the present invention contemplates that recombinant genes for glucan synthase or glucan synthase-related protein may be derived from eukaryotic cell lines 5 as well.

Commercial applications of microbially derived cellulose, from the bacterium Acetobacter xylinum for instance, has distinct advantages over traditional, non-10 microbial sources of this ubiquitous natural polymer. For instance, no delignification is required following harvest. Moreover, the product can be synthesized directly into an extremely strong non-woven textile of virtually any shape thereby reducing many steps in the 15 conversion of natural cellulose into a finished product. The invention provides a recombinant cellulose synthase or cellulose synthase-related protein whereby the physical properties of the product of the enzyme (cellulose) such as crystallinity, hydrophobicity, and degree of 20 polymerization can be controlled during synthesis. Most importantly, however, the present invention provides the unique possibility of producing commercially feasible levels of cellulose from a wide variety of substrates. It should be possible, using the recombinant gene of the 25 invention, to transform, for instance, photosynthetic, nitrogen-fixing prokaryotes (cyanobacteria) or single-celled eukaryotes (algae).

The ability to alter physical characteristics of the 30 cellulose in situ may provide an efficient method for producing a broad range of value-added products not possible before the recombinantly produced cellulose of the present invention was obtained. Previously, the development of a commercially feasible fermentation system 35 for producing cellulose from bacteria has presented a significant technological challenge. Even using the most

preferred bacterial strain, large-scale fermentation has been complicated by a number of phenomena associated with the biology of acetic acid bacteria including strain instability, the synthesis of gluconic acid as a 5 significant by-product, and poor oxygen diffusion through the cellulose.

The present invention overcomes most of the complications of the prior art by providing a means for 10 producing a recombinant  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein. The recombinant glucan synthase or cellulose synthase-related protein of the present invention allow production of a wide array of glucan polymer based products with significantly higher 15 efficiency than the natural sources of cellulose. In so doing, the present invention may make its greatest contribution by preserving valuable natural resources.

For instance, cellulose from the cell walls of woody 20 plants is formed in close association with lignin, hemicellulose, and other compounds. For many applications, such as in paper manufacture, wood cellulose must first be delignified by an extensive process known as pulping. Depending upon the process employed, pulping 25 requires four or five processing steps following logging to obtain a partially purified cellulose material (Kirk-Othmer Concise Encyclopedia of Chemical Technology 1985). In contrast, Acetobacter xylinum and the recombinant cellulose synthase or cellulose synthase-related protein 30 derived therefrom will be capable of synthesizing an extracellular ribbon of nearly pure cellulose directly into the incubation medium. The ribbons of individual bacterial cells interweave to form a hydrophilic cellulose membrane known as a pellicle. Alternatively, in vitro 35 product synthesis of glucan polymers may be varied using

the methods of the invention depending on the glucan synthase selected or the substrates provided.

5       The production of relatively high yields of Acetobacter cellulose without the corresponding synthesis of lignin and hemicelluloses greatly facilitates the process required to purify the cellulose. Nearly all of the cellular biomass and incubation medium components can be efficiently removed from the pellicle by a simple  
10      cleaning process. If necessary, the pellicle can then be dried by various means as required for a particular end product use. The simpler cleaning process of Acetobacter cellulose may result in higher end product yields with greater purity than can be obtained by the pulping process  
15      required for woody cellulose products.

20      The native Acetobacter pellicle has outstanding hydrophilicity and it is believed that the recombinant cellulose synthase and cellulose synthase-related protein will share this attribute. Depending upon synthetic conditions, it may have a water holding capacity ranging from 60 to 700 times its dry weight. The hydrophilicity of the cellulose pellicle is due in part to the extensive interior surface area of the interstitial spaces of the  
25      "never dried" pellicle/microfibril matrix. The presence of pore structures and "tunnels" within the wet pellicle may be the reason for its rapid uptake of post synthetic processing agents or dyes (Thompson 1988). Other sources of cellulose, such as natural cotton and wood enter the  
30      manufacturing process in a more compacted form. This form is not only less hydrophilic than microbial cellulose but is also less susceptible to chemical treatments or to dye uptake. As a consequence, harsher physical and chemical processing is required, reducing cellulose yields and  
35      increasing processing costs. Recombinant sources of these important enzymes will allow much improved products.

5        The strictly aerobic nature of Acetobacter may lend itself to industrial application. In undisturbed cultures, Acetobacter forms the cellulose pellicle at the air/liquid interface. Through the use of gas permeable molds, "microwoven" textiles of virtually any shape can be produced (U.S. Patent Application Serial No. 229,888 [REDACTED], Serial No. 684,844], filed April 17, 1989, Brown et al.). It has been demonstrated that a molded, seamless fabric in the shape of a glove can be formed *in situ* by an active

10      Acetobacter culture. These microbial textiles can be synthesized into extremely thin, pliable forms which are gas and liquid permeable. The recombinant cellulose synthase and cellulose synthase-related protein of the 15 present invention being derived from these bacterial cells will most likely share these unique advantages. Moreover, the significant advantage presented by the recombinant cellulose synthase and cellulose synthase-related protein will overcome the problems associated with the use of the 20 natural bacterium.

25      The native Acetobacter pellicle has mechanical properties, including shape retention and tear resistance, which are superior to many synthetic fibers. It has been reported that following heat and pressure treatment, microbial cellulose has a Young's Modulus (a measure of shape retention) of 30 giga-Pascals, 4 times greater than any organic fiber (The Japan Industrial Journal, May 15, 1987; Japanese New York Times, May 29, 1987). The same 30 report indicates that the tensile strength (tear resistance) of the treated material is five times greater than polyethylene or vinyl chloride films. Sony Corporation (Japan) has taken advantage of the shape retention properties of the treated Acetobacter cellulose 35 in the development of a high fidelity audio speaker diaphragm made from the material (The Japan Industrial

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Journal, May 15, 1987; Japanese New York Times, May 29, 1987). The Ajinomoto Company (Japan), a collaborator with Sony in the development of the cellulose treatment process, has begun distributing samples of ultrahigh 5 strength paper manufactured from Acetobacter cellulose (The Japan Industrial Journal, May 15, 1987; Japanese New York Times, May 29, 1987). These commercial applications are indicative of the types of applications which will benefit significantly from the current inventions 10 compositions and methods.

Perhaps the most significant advantage of the invention, however, stems from the possibility that it presents to produce cellulose from a wide array of 15 potential substrates. Acetobacter xylinum, itself, is capable of producing cellulose from a variety of inexpensive carbon substrates. Widely available commodity sources of carbon which may be utilized by Acetobacter include dextrose (glucose), sucrose, fructose, invert 20 sugar, ethanol and glycerol [Tarr and Hibbert (1934); Barsha and Hibbert (1934)]. This diversity of utilizable substrates provides considerable flexibility in the location of the manufacturing facility since at least one 25 of these substrates is produced in virtually every region of the world. Flexibility in the manufacturing process is enhanced by the ability to substitute among a variety of possible fermentation substrates and provides some independence from impact of price increases for an individual substrate. The ability to employ a substrate 30 diversification strategy is particularly advantageous in view of the significant price volatility associated with international agricultural commodity products.

In particular, with the methods and compositions of 35 the present invention, an even wider range of potential substrates becomes possible. This wide range of

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substrates is particularly advantageous when combined with the variable  $\beta$ -1,4 glucan synthases of the present invention. It is not unlikely that the recombinant genes disclosed herein when placed on the appropriate vector and 5 into the appropriate cell line, may be capable of directing the

photosynthetic, nitrogen fixing cell thereby converting carbon dioxide and water directly into the desired product. The substantial advantages represented by this 10 embodiment will be apparent to those of skill in the art.

A major advantage of the  $\beta$ -1,4 glucan biopolymer technology lies in the ability to control the physical characteristics of the native glucan biopolymer as it is 15 being synthesized. These advantages will be even more available utilizing the recombinant glucan synthase or glucan synthase-related protein of the invention.

Previous investigations have demonstrated that the addition of direct dyes such as Congo Red, fluorescent 20 brightening agents such as Tinopal LPW, and carboxycellulose derivatives to the Acetobacter culture can alter the assembly of the cellulose ribbons [Ben-Hayyim and Ohad (1965); Haigler et al. (1982)].

25 Depending upon the agents employed and their concentration, a variety of cellulose structures can be produced. The variation in these structures can range from individual microfibrils that are loosely associated into ribbons to completely disassociated fibrillar

30 structures, resulting in amorphous cellulose. The extension of this earlier research has resulted in the development of a proprietary process which can significantly effect the absorptive properties of Acetobacter cellulose. This process enables nearly 35 complete rehydration of the pellicle to its original hydrophilicity even after three cycles of wetting followed

by drying at elevated temperatures (U.S. Patent Application, Serial No. 022,904, filed March 6, 1987, Brown et al.). It may be possible that Acetobacter cellulose synthesized by this process can be used as a 5 reusable super-absorbent material. At any rate, the recombinant glucan synthase or glucan synthase-related protein of the present invention greatly enhances the already substantial benefits of these procedures.

10        The ability to control the glucan biosynthesis process within a closed system provides several advantages. It enables the manufacturer to alter the properties of the recombinant glucan such as cellulose to a greater degree than would be possible by post-synthetic 15 processing of other sources of cellulose. By manipulating the cell density, incubation conditions, fermentation vessel configuration, and media components, the opacity, strength and pliability of the harvested cellulose product can also be controlled. It is possible to control the 20 degree and type of polymerization of the cellulose as well (U.S. Patent 450,324, issued December 19, 1982).

25        The compositions and methods of the invention which now make possible these commercially desirable goals, are exemplified below. In order to achieve the considerable 30 advantages of the invention, purification of a  $\beta$ -1,4 glucan synthase and/or  $\beta$ -1,4 glucan synthase-related protein to a substantially purified state must first be accomplished. Next, the substantially pure glucan 35 synthase or glucan synthase-related protein is used in order to identify the catalytic subunit of a multiple subunit enzyme. Following this identification, the catalytic subunit is at least partially sequenced and used to design oligonucleotide probes for hybridizing to the DNA of a glucan synthase containing cell line. The resulting hybridizing DNA is cloned and used to derive a

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full length glucan synthase or glucan synthase-related protein gene from the DNA source.

5           In the case of cellulose synthase of *A. xylinum*, probes capable of identifying the cellulose synthase catalytic sequence were further useful in identifying the [REDACTED] sequence as well.

10           It is anticipated that other such operonic associations may be encountered in other  $\beta$ -1,4 glucan synthase systems. Once these steps have been accomplished, the substantial advantages provided by the compositions of the invention including the substantially purified protein and the DNA segments may be used in a wide range of applications and embodiments including transformation of cells with the 15 recombinant cellulose synthase or cellulose synthase-related protein and use of the DNA probes of the invention to probe for similar genes in other DNA sources including eukaryotic plant DNAs.

20           Summary of Purification Studies

25           Beyer et al. (1981) presented several problems that must be overcome if a glycosyltransferase is to be successfully purified. These included: (i) selection of a cell strain which can produce a high yield of active transferase; (ii) an efficient harvest of viable cells from a selected culture method; (iii) a factor for stabilization of the enzyme; (iv) choice of the detergent; and, (v) an efficient purification procedure.

30           During the course of purification of cellulose synthase, the present inventors encountered a number of similar obstacles as well as ones unique to the glucan synthases. Several strategies had to be developed in order to overcome these problems. First, an *A. xylinum* strain ATCC 53582 was selected since it has a greater

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enzyme activity than ATCC 23769. Second, a method for production of high yields of essentially cellulose-free Acetobacter cells using cellulase was refined (Dillingham (1961); U.S. Patent Application, Serial No. 900,384, filed 5 August 26, 1986, Brown et al.). Third, Triton X-100 was selected as the detergent and glycerol was selected as the enzyme stabilizer.

10 The present inventors tested various purification methods, including sucrose or glycerol gradient ultracentrifugation, gel filtration with Sephacryl S-300, metal chelating affinity chromatography, anion-exchange chromatography with DEAE-Sepharose CL-6B, and affinity chromatography with UDP-glucuronic acid-agarose. However, 15 all either failed or resulted in only a 2-3 fold purification of the synthase.

20 A high degree of purification was achieved by in vitro cellulose-entrapment. The reason for such a high degree of purification being achieved is unclear. However, it could be due to: (a) the enzyme may be tightly enclosed by its insoluble product and thus coprecipitates as speculated by Kang et al. (1984) for chitin synthetase; (b) the density of cellulose is high, 25 about 1.27-1.6 (*CRC Handbook of Chemistry and Physics*. 1984), which allows the enzyme product complex to pass through the 30% glycerol layer; and\or, (c) the 30% glycerol content of the cushion solution keeps most of the protein's in the reaction mixture during centrifugation.

30

35 It has been reported that three different subunits of the photosystem II reaction center could be isolated using Triton X-100, whereas five different subunits were obtained using digitonin (Nanba and Satoh 1987). Both purified preparations have the same photochemical activity. From these observations, the inventors proposed

that different detergents might solubilize an oligomeric enzyme complex in association either with or without non-essential proteins, even after purification. This may also be the case with the purification of cellulose synthase using different detergents. Under reducing conditions, the 83 Kd and 92 Kd bands

10 was degraded and only the 83 Kd band remained if the purified preparation was treated with trypsin. These results, in combination with the evidence from the characterization of cellulose synthase as a glycosylated protein, suggested that the 83 Kd band is an essential part of the active cellulose synthase. However, the possible involvement of the 93 Kd polypeptide with the cellulose synthase could not be excluded since the enzyme may consist of non-identical subunits. In addition, the presence of the other protein bands in the most purified preparations was still a considerable concern. Definitive identification of the subunit composition of cellulose synthase had to await further investigation.

As shown in Fig. 4 (lane 1 and 2) which is described more fully hereafter, the 93 Kd band observed under reducing conditions and a 101 Kd band seen under non-reducing conditions could be the same polypeptide with intramolecular disulfide bonds. The presence of two small spots in the position of the 93 Kd band under non-reducing conditions (Fig. 4, lane 1, arrowheads), may be due to the partial degradation of intramolecular disulfide bonds during the preparation of the enzyme fraction. This also may explain the occurrence of two peaks present in the gel filtration elution profiles. In any case, such extraneous bands increased considerably the difficulty associated with the identification of the catalytic cellulose synthase polypeptide.

Dennis and Colvin (1965) noted that incubation of lysed cell preparations with trypsin at room temperature for 24 hours completely removed the cytoplasmic membrane and associated materials, and completely inhibited 5 cellulose synthesis. They suggested that the site of synthesis might be the cytoplasmic membrane. Recently, Bureau and Brown (1987) also suggested that the cellulose synthase is located on the cytoplasmic membrane. However, their studies showed that cellulose synthase activity is 10 maintained following brief trypsin treatment. Trypsin is well known for degrading proteins and also is effective for the cleavage of the peptidoglycan attachment region of lipoprotein in Gram-negative bacteria (Braun 1975).

15 In studies disclosed herein, trypsin was found to have no effect on cellulose synthase activity and resulted in more efficient detergent solubilization of the enzyme. However, it was not clear: (i) why trypsin degraded the solubilized form of the 93 Kd polypeptide, but did not 20 degrade the membrane-bound form; or (ii) why the specific activity of the peak fraction from trypsin treated (TS; triton solubilized) is twice as great as the non-treated sample in gel filtration chromatographic studies. Elucidation of the interaction between trypsin and 25 cellulose synthase required more study. Certainly, however, the ability to purify cellulose synthase provides a basis for certain surprising aspects of the invention. These techniques represent the first instance of the substantial purification of the cellulose synthase from *A. xylinum*, in particular, or any  $\beta$ -1,4 glucan synthase, in 30 general.

## Summary of the Studies Designed to Identify the Active Subunit of a Glucan Synthase

5 By using a photoaffinity labeling method, the substrate binding subunit of cellulose synthase, e.g., from Acetobacter xylinum strain ATCC 53582, was shown to

10 the findings of others which identify an 85.3 Kd polypeptide, homologous to the 93 Kd polypeptide of the present invention, as the catalytic subunit. This 93 Kd polypeptide may include or comprise a regulatory subunit of cellulose synthase (i.e., the c-di-GMP binding subunit). Conversely, the 83 Kd polypeptide may be strictly a catalytic subunit with regulatory functions 15 segregated into a distinct polypeptide, such as the 93 Kd polypeptide. In fact, there exists circumstantial evidence that the 93 Kd polypeptide is the effector-binding subunit (Mayer et al. 1989). Thus, the possible involvement of the 93 Kd polypeptide in the cellulose 20 synthase complex cannot yet be excluded since the enzyme complex may consist of non-identical subunits for various functions in the polymerization and crystallization of cellulose (Lin and Brown 1989).

25        The 83 Kd polypeptide had been partially  
characterized as a glycoprotein by lectin affinity  
chromatography, Schiff-periodate and fluorescein  
isothiocyanate-Concanavalin A staining analyses (Lin and  
Brown 1989). However, since membrane associated  
30        glycoproteins have been widely found to be difficult to  
purify, the finding that [ $\beta$ -<sup>32</sup>P]5N<sub>3</sub>UDP-glc binds to the 83  
Kd catalytic subunit of cellulose synthase in *A. xylinum*  
provided the present inventors with a powerful tool for  
elucidation of the structure, function, and regulation of  
35        the cellulose synthase. Furthermore, it provided one  
means for the isolation of recombinant genes encoding the

5 catalytic 83 Kd polypeptide as well as the 93 Kd polypeptide immediately downstream. Only upon purification did it become feasible to sequence the polypeptide and construct an oligonucleotide probe for cloning.

10 It is also important to note here that the present inventors have applied the techniques of the invention to another wild type strain of *A. xylinum* known to produce cellulose I pellicles, ATCC 23769, and to a pellicle-free mutant derived from the ATCC 53582 strain (called NQ5). In so doing, the present inventors identified a 75-Kd polypeptide as the catalytic subunit of cellulose synthase which is in contrast to the 83-Kd subunit from ATCC 53582. 15 Thus, the techniques described herein, are generally applicable for identifying the catalytic subunit of purified cellulose synthases in various bacterial strains.

20 Furthermore, the azido probe techniques of the invention coupled with incorporation of radioactive UDP-Glu into glucan product have been demonstrated to be effective in identifying glucan synthases in the fiber-producing cells of cotton which make  $\beta$ -1,4 and  $\beta$ -1,3 glucan.

25

#### Cloning and Sequencing studies

30 Thus, the present invention provides a predictably successful, albeit difficult, method for the purification of glucan synthase made feasible by the techniques discussed above. Using solely prior art techniques, others had failed to obtain glucan synthase of adequate purity and quantity to enable either the identification of the active subunit or the sequencing of the active subunit 35 polypeptide. Furthermore, considerable disagreement exists in the art concerning the size and immunological

characteristics of the catalytic subunit of the cellulose synthase.

Application of cellulose entrapment, use of the cellulose synthase activator to enhance cellulose synthase activity, and photoimmobilization, identified above, among other techniques, all combined to allow the present inventors to supersede the prior art and go on to sequence the glucan synthase polypeptides and, ultimately, to clone the genes encoding them. It will be appreciated by those of skill in the art that once provided with the techniques for obtaining a substantially purified glucan synthase, and with the amino acid sequences of the present invention, many of the difficult steps necessary for cloning and sequencing the gene encoding a glucan synthase polypeptide have been overcome by the present invention.

Armed with the highly purified catalytic subunit of cellulose synthase, the present inventors were poised to utilize the techniques of molecular biology to clone and sequence the genes associated with cellulose synthase. However, considerable difficulties were encountered even at this stage.

Initially, the inventors attempted to obtain the amino acid sequence of the purified enzyme and failed. Only an ambiguous sequence resulted from these studies. However, upon proteolytic treatment with trypsin, a relatively non-ambiguous sequence was obtained. Based on the unambiguous sequence of the invention, eight 17-mer oligonucleotide were prepared for use as probes.

These probes were each used independently to determine which probe exhibited strongest binding potential when tested against total DNA derived from A.

5           xylinum. This technique was a substantially high-risk experiment since even strains of E. coli not known to contain a cellulose synthase gene sequence were found to strongly bind to certain of the probes. Therefore, there was certainly no guarantee that any of these probes, even if one should demonstrate binding potential to A. xylinum, would be capable of hybridizing to the actual cellulose synthase DNA.

10           In fact, a further complication arose from the very demonstration that E. coli strains known to those of skill in the art readily bound the probes of the invention. Since these probes would most likely not be capable of detecting a recombinant gene sequence in such a 15 background, the inventors were forced to forgo the typical technique applied by those of skill in the art, that technique being the creation of a total genomic DNA library based on the genomic DNA from A. xylinum. Instead, the inventors were forced to rely on the much 20 less certain technique of using the selected probe to identify bands of hybridizing DNA from a gel containing total genomic A. xylinum DNA which had been treated with restriction endonuclease.

25           This only allowed the isolation of a certain subset of DNA which might hybridize specifically with the probe, so the resulting DNA was subcloned into a vector and used to transform host bacteria. Even further difficulties were encountered at this point since only very few 30 recombinants were obtained, most likely due to the restriction of the A. xylinum heterologous DNA in E. coli. Additionally, since it was not possible to screen the host bacteria due to a high background of probe binding even under stringent conditions, the recombinant plasmids had 35 to be reisolated from the clones, pooled into 50 isolates per group and tested once again with the probe DNA for

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groups which demonstrated binding. Only after the application of these multiple-step techniques were the inventors herein able to isolate the recombinant plasmid which contained the hybridizing DNA, to subclone 5 individual fragments of this DNA to the point that they contained substantially only the sequence encoding

resulting fragments were utilized to obtain a DNA sequence of 2912 base pairs which was a region of the DNA sequence 10 encoding a protein consisting of 723 amino acid residues.

As noted previously, it is believed that modification and changes may be made in the structure of the recombinant glucan synthase protein and still obtain a 15 molecule having like or otherwise desirable characteristics. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with complementary structures such as antigen-binding regions of antibodies (e.g., binding sites on receptor 20 molecules). Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or, of course, its underlying DNA coding sequence) and, nevertheless, obtain a protein with like or even 25 countervailing properties (e.g., antagonistic v. agonistic). It is thus contemplated by the present inventors that various changes may be made in the sequence 30 of the peptides (or underlying DNA) without appreciable loss of their biological utility or activity.

Biological Functional Equivalency

35 The importance of the hydropathic index of amino acids in conferring interactive biologic function on a

protein has been discussed generally by Kyte and Doolittle (1982) wherein it is found that certain amino acids may be substituted for other amino acids having a similar hydrophobic index or score and still retain a similar biological activity. As displayed in Table I below, amino acids are assigned a hydrophobic index on the basis of their hydrophobicity and charge characteristics. It is believed that the relative hydrophobic character of the amino acid determines the secondary structure of the resultant protein, which in turn defines the interaction of the protein with substrate molecules. It is proposed that functional equivalency may typically be maintained where amino acids having no more than a +/- 1 to 2 difference in index, and more preferably within about a +/- 1 difference, are exchanged.

TABLE I

	<u>Amino Acid</u>	<u>Hydropathic Index</u>
20	Isoleucine (Ile)	4.5
	Valine (Val)	4.2
	Leucine (Leu)	3.8
	Phenylalanine (Phe)	2.8
	Cysteine/cystine (Cys)	2.5
25	Methionine (Met)	1.9
	Alanine (Ala)	1.8
	Glycine (Gly)	-0.4
	Threonine (Thr)	-0.7
	Tryptophan (Trp)	-0.9
30	Serine (Ser)	-0.8
	Tyrosine (Tyr)	-1.3
	Proline (Pro)	-1.6
	Histidine (His)	-3.2
	Glutamic Acid (Glu)	-3.5
35	Glutamine (Gln)	-3.5
	Aspartic Acid (Asp)	-3.5

Asparagine (Asn)	-3.5
Lysine (Lys)	-3.9
Arginine (Arg)	-4.5

5 Thus, for example, isoleucine, which has a  
hydropathic index of +4.5, can be substituted for valine  
10 having similar biologic activity. Alternatively, at the  
other end of the scale, lysine (-3.9) can be substituted  
for arginine (-4.5), and so on.

Accordingly, these amino acid substitutions are generally based on the relative similarity of R-group substituents, for example, in terms of size, electrophilic character, charge, and the like. In general, exemplary substitutions which take various of the foregoing characteristics into consideration include the following:

20

TABLE II

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>
25	Ala	gly; ser
	Arg	lys
	Asn	gln; his
	Asp	glu
	Cys	ser
	Gln	asn
	Glu	asp
30	Gly	ala
	His	asn; gln
	Ile	leu; val
	Leu	ile; val
	Lys	arg
35	Met	met; leu; tyr
	Ser	thr

Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

5

site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, derived from the cellulose synthase sequence, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art as exemplified by publications such as Adelman, et al. (1983), incorporated herein by reference. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing et al. (1981), incorporated herein by

reference. These phage are readily commercially available and their use is generally well known to those skilled in the art.

5 In general, site-directed mutagenesis in accordance  
herewith is performed by first obtaining a single  
10 sequence a DNA sequence  
which encodes the glucan synthase sequence. An  
oligonucleotide primer bearing the desired mutated  
sequence is prepared, generally synthetically, for example  
15 by the methods described herein. This primer is then  
annealed with the singled-stranded vector, and subjected  
to DNA polymerizing enzymes such as E. coli polymerase I  
Klenow fragment, in order to complete the synthesis of the  
mutation-bearing strand. Thus, a heteroduplex is formed  
20 wherein one strand encodes the original non-mutated  
sequence and the second strand bears the desired mutation.  
This heteroduplex vector is then used to transform  
appropriate cells such as E. coli cells and clones are  
selected which include recombinant vectors bearing the  
mutated sequence arrangement.

### Host Cell Cultures and Vectors

25 In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and constructing the vectors useful in the invention. For example, E. coli. HB101 has been shown to be particularly useful. Other microbial strains which may be used include E. coli strains such as E. coli B, and E. coli X 1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

35 Prokaryotes may also be used for expression. The aforementioned strains, as well as E. coli W3110 (F-, lambda-prototrophic bacilli such as Bacillus subtilis, or

other enterobacteriaceae such as Salmonella typhimurium or Serratia marcesans, and various Pseudomonas species may be used.

5        In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of 10 providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species and well known to those of skill in the art. The vector pBR322 contains genes for ampicillin and tetracycline resistance and thus 15 provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins.

20        Those promoters most commonly used in recombinant DNA construction include the  $\beta$ -lactamase (penicillinase) and lactose promoter systems and a tryptophan (trp) promoter system each of which is well known to those of skill in the art. While these are the most commonly used, other 25 microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors.

30        In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. Saccharomyces cerevisiae, the Agro or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a 35 number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for

example, is commonly used. This plasmid already contains the trpl gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1. The presence of the trpl lesion as a characteristic of the yeast host cell genome then provides a selection marker.

absence of tryptophan.

10                    Suitable promoting sequences in yeast vectors include  
the promoters for 3-phosphoglycerate kinase or other  
glycolytic enzymes [Hess et al. (1968); Holland et al.  
(1978)], such as enolase, glyceraldehyde-3-phosphate  
dehydrogenase, hexokinase, pyruvate decarboxylase,  
15                    phosphofructokinase, glucose-6-phosphate isomerase, 3-  
phosphoglycerate mutase, pyruvate kinase, triosephosphate  
isomerase, phosphoglucose isomerase, and glucokinase. In  
constructing suitable expression plasmids, the termination  
sequences associated with these genes are also ligated  
20                    into the expression vector 3' of the sequence desired to  
be expressed to provide polyadenylation of the mRNA and  
termination. Other promoters, which have the additional  
advantage of transcription controlled by growth conditions  
are the promoter region for alcohol dehydrogenase 2,  
25                    isocytochrome C, acid phosphatase, degradative enzymes  
associated with nitrogen metabolism, and the  
aforementioned glyceraldehyde-3-phosphate dehydrogenase,  
and enzymes responsible for maltose and galactose  
utilization. Any plasmid vector containing a yeast-  
30                    compatible promoter, origin of replication and termination  
sequences is suitable.

## Cotton Transformation

35 An example is provided which illustrates the manner  
in which an agronomic or other eukaryotic plant species

may be transformed with recombinant  $\beta$ -1,4 glucan synthase. It is based on the successful transformation of cotton (Gossypium hirsutum) with a recombinant gene for kanamycin resistance from bacteria, a situation not unlike that 5 which the present invention now makes possible for transformation of plant cells with, for instance, bacterial cellulose synthase. It will be recognized by those of skill in the art, that there are certain 10 limitations unique to the glucan synthases of the present invention which may not be addressed in the example below. However, the example demonstrates that there are not insurmountable obstacles to transforming a cotton plant with a glucan synthase such as a bacterial cellulose synthase.

15 The technique basically utilizes a binary plant vector strategy based on the Agrobacterium tumefaciens Ti plasmid. It further involves the cloning of the glucan synthase gene in a plant transformation vector (PTV). 20 This modified vector is then mated from E. coli into the Agrobacterium and recombinant colonies are selected which contain the recombinant gene. The Agrobacterium strain carries a "disarmed" Ti-plasmid. Virulence functions on the Ti-plasmid interact in trans with the border sequence 25 on the PTV thereby mobilizing the glucan synthase gene into the plant cell and inserting the gene into one of the host plant's chromosomes. Callus derived from these transformed cotyledons is treated with various plant hormones and stimulated to regenerate whole recombinant 30 plants according to techniques known to those of skill in the art.

There are, of course, a variety of methods known to 35 those of skill in the art for introducing recombinant genes into plants as well as a number of plant species into which recombinant DNA has been introduced (see

generally, Gasser and Fraley 1989). In those systems where Agrobacterium-mediated transformation is efficient, such as in cotton as noted herein, it is the method of choice because of the ease and defined nature of the gene transfer system utilized. However, few monocotyledonous plants appear to be hosts for Agrobacterium, thus

delivery systems for recombinant DNA into these species.

10        Certain of these techniques rely on physical means of introducing DNA into the plant cell. Transformation of protoplasts has been achieved through facilitating DNA uptake by calcium phosphate precipitation, polyethylene glycol treatment, electroporation, or combinations of these methods. These techniques rely on introduction of recombinant DNA into protoplasts of the plant cells. Where regeneration of protoplasts has been shown to be difficult, mechanical means to introduce recombinant DNA into intact cells or tissues has been successfully used.

15        In particular, the use of particle guns (high-velocity microprojectile technology) has been shown to affect transformation of plant cells. Other methods with the potential to achieve plant cell transformation with recombinant DNA include transfer into pollen, direct injection into reproductive organs, microinjection into cells of immature embryos and rehydration of dessicated embryos.

20

25

Furthermore, a growing number of species have been reported from which the production of transgenic plants has been achieved with one or another of the techniques noted above. The following is a recently updated list of such plants and a reference to the method used to affect the transformation (see generally, Gasser and Fraley 1989: Abbreviations; AT, Agrobacterium tumefaciens; AR Agrobacterium rhizog nes; FP, free DNA introduction into

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protoplasts; PG, particle gun; MI, microinjection; IR, injection of reproductive organs).

	<u>PLANT SPECIES</u>	<u>METHOD</u>
5	<i>Herbaceous Dicots</i>	
	Petunia	AT
	Tomato	AT
	Potato	AT
	Tobacco	AT, FP, PG
10	<i>Arabidopsis</i>	AT
	Lettuce	AT
	Sunflower	AT
	Oilseed Rape	AT, MI
	Flax	AT
15	Cotton	AT
	Sugarbeet	AT
	Celery	AT
	Soybean	AT, PG
	Alfalfa	AT
20	<i>Medicago varia</i>	AT
	Lotus	AT
	<i>Vigna aconitifolia</i>	FP
	Cucumber	AR
	Carrot	AR
25	<i>Cauliflower</i>	AR
	Horseradish	AR
	Morning Glory	AR
	<i>Woody Dicots</i>	
30	Poplar	AT
	Walnut	AT
	Apple	AT
35	<i>Monocots</i>	
	Asparagus	AT
	Rice	FP
	Corn	FP
40	<i>Orchard Grass</i>	FP
	Rye	IR

45 Plant DNA Hybridization

Another example is provided to illustrate a proposed use of the recombinant glucan synthase of the present invention to probe plant DNA sources for hybridizing sequences. This method allows for a rapid microscale isolation of plant DNA without the use of CsCl gradients.

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The technique was developed for Zea mays but has been successfully applied to a variety of other plant species including Nicotiana tabacum, N. plumbaginifolium, N. sylvestris, Lycopersicum sp., Amaranthus sp., Glycine max, 5 and Petunia hybrida.

tissue DNA from leaf tissue by disrupting the cells of the leaf frozen in liquid nitrogen. The disrupted cell debris is then solubilized in detergent and buffer in order to remove most of the proteinaceous and polysaccharide materials. After filtering the debris from the buffered solution, plant DNA is then precipitated with alcohol, dried and resuspended in a buffered solution, then 15 precipitated using a high salt-alcohol method. The resulting DNA may be used with restriction endonucleases and other DNA-modifying enzymes. It has also been demonstrated to be useful in filter hybridization using DNA probes such as those of the present invention.

20 As mentioned, in certain aspects, the DNA sequence information provided by the invention allows for the preparation of relatively short DNA (or RNA) sequences having the ability to specifically hybridize to gene 25 sequences of the glucan synthase gene. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of the sequence shown in Figure 1 or Figure 13. The ability of such nucleic acid probes to specifically hybridize to the glucan synthase gene 30 sequences lend them particular utility in a variety of embodiments. Most importantly, the probes can be used in a variety of assays for detecting the presence of complementary sequences in a given sample such as the plant DNA samples described here. However, other uses are 35 envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers

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for use in preparing other genetic constructions such as for obtaining full length clones of sequences downstream or upstream of the original clones of the invention.

5        To provide certain of the advantages in accordance with the invention, the preferred nucleic acid sequence employed for hybridization studies or assays includes sequences that are complementary to at least a 14 to 25, or so, nucleotide stretch of the sequence shown in Figure 10      1 or Figure 13. A size of at least 14 nucleotides in length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both 15      stable and selective. Molecules having complementary sequences over stretches greater than 10 bases in length 20      are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches 25      of 14 to 20 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102, or by introducing selected sequences into recombinant vectors for recombinant production.

30        Certain nucleotide sequences of the invention will be important for their ability to selectively form duplex molecules with complementary stretches of the gene. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degree of selectivity of the probe toward the target sequence. For applications requiring a high degree 35      of selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, for

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example, one will select relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M NaCl at temperatures of 50°C to 70°C. These conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate cellulose synthase coding sequences for related species, functional equivalents, or the like, less stringent hybridization conditions are called for in order to allow formation of the heteroduplex. In these circumstances, one would desire to employ conditions such as 0.15M-0.9M salt, at temperatures ranging from 20°C to 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal.

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In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, 5 the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular 10 circumstances based on the particular criteria required (depending, for example, on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound 15 probe molecules, specific hybridization is detected, or even quantified, by means of the label.

While it is not specifically addressed in this example, the use of the methods and the compositions of 20 the present invention may be applicable in certain instances to non-plant eukaryotes. In particular, oriented cellulose has been shown to be a component of certain mammalian tissues. There is considerable evidence that polysaccharides play an important role in the 25 stabilization of connective tissue fibers and as such may play a role in certain disease conditions in humans such as scleroderma conditions [see generally, Hall et al., *Proc. Royal Soc. B* 151; 497 (1960)].

30 In any event or from whatever source, once the DNA fragments have been detected via these techniques, standard approaches known well to those of skill in the art may be utilized to further isolate and clone the fragments. As alternative sources of recombinant glucan 35 synthases, these new fragments will share all the

utilities of the compositions described specifically herein.

Transformation of Cyanobacteria with Cellulose Synthase

5

Cyanobacteria (blue-green algae)

What do cyanobacteria and plants have in common? They are prokaryotes and they carry out oxygenic, plantlike photosynthesis. Many strains of cyanobacteria are also capable of fixing atmospheric nitrogen. These properties make cyanobacteria prime candidates for transformation with recombinant glucan synthase genes from A. xylinum in order to develop commercial scale glucan biopolymer production in a prokaryotic species capable of photosynthesis and nitrogen fixation. This is particularly attractive with regard to cellulose biosynthesis.

Typically, no cellulose biosynthesis has been demonstrated in cyanobacteria under normal growth conditions. However, cellulose produced in the form of a slime has been reported in Nostoc [A. Frey-Wyssling, The Plant Cell Wall, p.227, Gebruder Borntrager, Berlin (1976)]. The transfer of an A. xylinum cellulose synthase gene into cyanobacteria is made possible with the methods and compositions of the present invention.

Gene transfer has been established and host-vector systems have been developed for gene cloning in Cyanobacteria (Kuhlemeier and Van Arkel 1987). Two strategies have been employed for gene cloning in Cyanobacteria. One uses shuttle plasmid vectors that carry both a cyanobacterial and an E. coli replicon, each of which functions in its indigenous host. Depending on the sequence of the cloned gene, the selection applied, and the cyanobacterial host, the cloned gene can be

maintained on the autonomous plasmid or can be integrated into the cyanobacterial chromosome.

5       The other strategy exploits the efficient recombination system of certain cyanobacterial strains (Anacystis nidulans R2, Synechocystis sp. 6803) for a directed integration of the cloned gene into the chromosome (Golden et al. 1987). In the latter strategy a DNA vector that lacks a cyanobacterial replicon, but 10 carries similar chromosomal sequences, is used. According to the imposed selective conditions, the cloned gene, either alone or together with the vector sequences, integrates into the chromosome.

15       Gene transfer in cyanobacteria is known to take place by conjugation and transduction as well as by transformation (Herdman 1982). Up to now, however, the only system of practical importance has been the transformation system (Porter 1988) and that is the 20 preferred method described herein.

25       A number of bacterial antibiotic resistance genes are expressed in cyanobacteria, where they are used for selecting transformed cells (Golden et al. 1987). Apart from these, the larvicultural gene of Bacillus sphearicus 1593M was transformed into the cyanobacterium Anacystis nidulans R2, where it exhibited expression and toxin activity comparable to that observed with E. coli carrying the respective recombinant plasmids (Tandeau de Marsac et 30 al. 1987). Use has also been made of the coding sequences of cat, lacZ, and lux to function as reporter genes in determining efficient promoter sequences (Friedberg 1988). The gene products of these genes can be assayed in 35 cyanobacteria allowing scoring or selection of cells expressing the gene.

EXAMPLE I: PURIFICATION OF CELLULOSE SYNTHASE

Materials - Reagents were obtained from the following sources: UDP-[<sup>14</sup>C]glc, lithium dodecyl sulfate, ICN; 5 polyethylene glycol (PEG)-3350, Triton X-100, n-octyl glucoside, trypsin (EC 3.4.21.4) trypsin inhibitor, [REDACTED] mannopyranoside, dansyl hydrazine, fluorescein isothiocyanate-labeled Con A (FITC-Con A), Sigma; 10 digitonin, Serva; Celluclast, Novo Enzymes; guanosine-5'-triphosphate (GTP) dilithium salt, Boehringer Mannheim. All operations were performed at 0-5°C, unless otherwise indicated.

Cells - Two bacterial strains of A. xylinum, ATCC 53582 and ATCC 23769, were used. Cultures were maintained on Schramm and Hestrin's glucose medium as previously described (Schramm and Hestrin 1954). A single colony, 15 grown for 5-7 days at 28°C on agar plates, was transferred to a flask containing 200 ml of culture medium and 75 µl filtered Celluclast, and cultured at 28°C on a rotary shaker set at 120 rpm for 42-48 hour [Dillingham (1961); U.S. Patent application No. 900,384, filed August 26, 20 1986]. A 5 ml aliquot of the starting culture was then transferred to a new flask having the same amount of medium and Celluclast and cultured under the same 25 conditions. After 48 hours, the cell suspension was passed through 8 layers of cheesecloth and then centrifuged at 10,000 x g for 10 minutes. The pellets were washed once in a buffer of 50 mM Tris-10mM MgCl<sub>2</sub>-1 mM EDTA, pH 7.5 (TME) as described by Glaser (Glaser 1958). The yield was about 1 gm of cells, dry weight, per liter 30 of culture. The washed cell pellets were resuspended in TME containing 20% (weight/ volume) PEG-3350.

Preparation of Membranes and Activator - "Washed membranes" and "crude factor" were prepared as described previously (Ross 1986) except that the cells were disrupted in an Aminco French pressure cell at 16,000 5 lb/in<sup>2</sup>. Trypsinized membranes were prepared as follows: to 10 ml of washed membranes (protein concentration, 5 mg/ml), 0.2 mg of trypsin was added and gently shaken at 25°C for 30 minutes. After the addition of 0.2 mg of trypsin inhibitor for 20 minutes, the mixture was 10 centrifuged at 18,000 x g for 20 minutes and washed once in TME.

Cyclic diguanylic acid, an activator of the cellulose synthase (Lin et al. 1985), was obtained in two ways as 15 described (Ross 1986), but with several modifications: (i) 8 ml of crude factor was mixed with 1 ml 10 mM GTP, 0.1 ml 100 mM CaCl<sub>2</sub> and 0.9 ml TME buffer, and incubated at 37°C for two hours. The reaction was terminated by heating in boiling water for three minutes, followed by 20 centrifugation at 2,000 x g for 10 minutes. This supernatant was called "crude Gx"; (ii) the activator was prepared as in (i) except that the reaction was terminated by addition of 0.6 M HClO<sub>4</sub> instead of heating. After 25 centrifugation, the supernatant was neutralized with 5 M KOH and recentrifuged at room temperature. This supernatant was designated "N-Gx."

Solubilization by Nonionic Detergent - Either the washed membranes or the trypsinized membranes were resuspended in 30 TME containing 20% glycerol, 0.6% (weight/volume) Triton X-100 to give a final protein concentration of 5.0 mg of membrane protein per ml. After homogenization with a Teflon homogenizer, the suspension was gently shaken for one hour, followed by centrifugation at 100,000 x g for 35 one hour. The supernatant represented the Triton-solubilized enzyme which was designated "TS" if the washed

membranes were used, and "TSt" if the trypsinized membranes were used. The n-octyl glucoside-solubilized enzyme (OGS and OGSt are equivalent to TS and TSt) was prepared as TS except that 1% (weight/volume) n-octyl glucoside was used with a two hour shaking time. The digitonin-solubilized enzyme (DS) was prepared as

Cellulose Entrapment Method - The entrapment method used was described earlier (Kang 1984), with the following modifications: cellulose nitrate tubes, 9/16 x 3 3/4 in., were used. A 2.6 ml of cushion solution, consisting of TME buffer and 30% glycerol (v/v), were added to the bottom of the tubes. Subsequently, a mixture of 6.8 ml of the solubilized enzyme, 0.2 ml of 100 mM UDP-glucose and 3.4 ml of N-Gx, was layered on the top of cushion solution. The tubes were incubated in a 30°C water bath for 11 minutes, then were placed on ice for two hours, and centrifuged for 20 minutes in a Beckman SW 40 Ti rotor at 50,000 x g. After overnight storage at -80°C, each pellet was resuspended in extraction buffer to 1/30 the original loading volume and homogenized with a Teflon homogenizer. Extraction buffer was prepared with 0.1% detergent in TME buffer containing 20% glycerol. The suspension was centrifuged for 10 minutes at 15,000 x g. The supernatant was designated ES<sub>1</sub> and the pellet, EP<sub>1</sub>. The EP<sub>1</sub> was resuspended in extraction buffer to the same volume as ES<sub>1</sub>.

30 ES<sub>2</sub> and EP<sub>2</sub> were the resulting supernatant and pellet respectively, from a second entrapment step of ES<sub>1</sub>. All purified enzymes were stored at -80°C.

35 Con A-Sepharose Chromatography - Triton X-100 solubilized  
enzyme pr paration, TS, was applied at a flow rate of one  
column volume/hour to a column (1 x 10 cm) of Con A-

Sepharose 4B previously equilibrated in an "enzyme buffer" of TME, 1 mM MnCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 20% glycerol and 0.1% Triton X-100. The flow was stopped after the entire sample was applied and allowed to stand for one hour. The 5 column was washed with 25 ml of enzyme buffer, and the bound protein was then eluted with 0.3 M methyl alpha-D-manno-pyranoside in enzyme buffer. One ml fractions were collected for enzyme assay.

10 Sephacryl S-300 Chromatography - A column (1.6 x 85 cm) of Sephacryl S-300 was equilibrated with TME buffer containing 0.1% Triton X-100 and 20% glycerol. The enzyme preparation, TS, was applied and eluted with the same buffer, at a flow rate of 11.0 ml/hr. Fractions of 3 ml 15 were collected and assayed for enzyme activity. The column was calibrated with a series of molecular weight markers (Pharmacia), usually Blue Dextran 2000, thyroglobulin, ferritin, catalase, aldolase and albumin.

20 Enzyme Assay - Assays contained a final volume of 0.2 ml: 20  $\mu$ l of 0.5 M Tris-HCl (pH 9.6) containing 50 mM MgCl<sub>2</sub>, and 5 mM EDTA, 20  $\mu$ l of 0.1 M MgCl<sub>2</sub>, 20  $\mu$ l of 10 MM CaCl<sub>2</sub>, 25  $\mu$ l of TME buffer, 25  $\mu$ l of H<sub>2</sub>O, 20  $\mu$ l of crude Gx, and 50  $\mu$ l of enzyme sample. Incubation was carried out in a 25 30°C water bath for either (a) 5 minutes in the final concentration of 20 $\mu$ M (110 cpm/pmol) UDP-[<sup>14</sup>C]glc or (b) 30 minutes in the final concentration of 1 mM (2.2 cpm/pmol) UDP-[<sup>14</sup>C]glc. The reaction was stopped with 2 ml of 0.5 M NaOH, and the radioactivity in alkali- 30 insoluble materials was counted after filtration through glass-fiber filters, as described in detail (Bureau and Brown 1987).

35 Protein D termination - Protein concentrations were determined according to the method of Bradford (1976), using the protein assay kit of Bio-Rad Laboratories. A

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modification of the Lowry procedure (Markwell 1978) was used only with membrane preparations. In both cases, bovine serum albumin was used as a standard.

5 Polyacrylamide Gel Electrophoresis - The samples were dissolved in sample buffer containing 2% lithium dodecyl

mercaptoethanol and 20% glycerol with 0.02% bromophenol blue. The incubation was carried out on ice for 1-2 hours, instead of boiling water for 3-5 minutes, because the heating frequently caused the purified protein bands on the gel to smear. Substitution of LDS for sodium dodecyl sulfate resulted in greater resolution for the purified enzyme preparations. Discontinuous 15 LDS/polyacrylamide gel electrophoresis (LDS-PAGE) was performed as described by Laemmli (1970) using 1.5-mm slab gels containing 8% acrylamide. The gels were stained with Coomassie brilliant blue or silver stained (Wray 1981). The molecular mass standards (Biorad) were phosphorylase b 20 (97 Kd), bovine serum albumin (66 Kd), ovalbumin (42 Kd), carbonic anhydrase (31 Kd), soybean trypsin inhibitor (21 Kd) and hen egg white lysozyme (14 Kd).

Photographs of Coomassie blue or silver stained gels 25 were digitized with an IBAS Interactive Image Analysis System (Zeiss, Thornwood, New York). The optical densities of the protein bands were measured by the image edit program.

30 Identification of Glycoproteins - Approximately 10  $\mu$ g protein of the EP<sub>1</sub> fraction purified from DS was separated by LDS-PAGE in an 8% gel. Subsequently, the periodic acid-Schiff base method using dansyl hydrazine (Eckhardt 1979) and FITC-labeled lectin method (Furlan 1979) were 35 respectively used for glycoprotein identification.

Purification of the Cellulose Synthase - Gel filtration, anion exchange or Con A-Sepharose chromatography were separately combined with the cellulose entrapment step for the purification of the cellulose synthase. However, the 5 recovery of enzyme activity was not high enough and the protein composition of the purified preparation was similar to that when directly purified by the cellulose entrapment (data not shown). Therefore, the cellulose entrapment method was used to purify the cellulose 10 synthase.

The detergent-solubilized enzyme preparation was mixed with the substrate and the enzyme activator. After incubation at 30°C for 11 minutes, the reaction mixture 15 was centrifuged, and most of the in vitro cellulose was sedimented. Enzyme activity was found primarily in the pellets. Generally, the ES<sub>1</sub> contained 20-30% of total protein in the pellets and showed only 10-15% of total enzyme activity. Over 80% of cellulose synthase 20 activities still remained in the EP<sub>1</sub>. Therefore, the EP<sub>1</sub> and EP<sub>2</sub> were used as the samples of purified preparations for further characterization.

Cellulose synthase from Acetobacter is very labile. 25 More than half of the activity of solubilized enzyme (such as DS, TS and OGS preparations) was lost after incubation, either at 4°C for 16 hours, or at 25°C for one hour. In contrast, entrapped enzyme (EP<sub>1</sub>) still maintains over half of its original activity after four hours at 25°C, while 30 the activities in the ES<sub>1</sub>, DS and TS are almost completely lost. It is suggested that the entrapment of the enzyme by the in vitro cellulose product has a stabilizing effect.

35 As shown in Table III, the purification in the first entrapment step was 42-fold for EP<sub>1</sub>. The second

entrapment showed a 2 to 3-fold purification, in comparison with EP<sub>1</sub>. The total purification in both steps was about 96-fold. The recovery of cellulose synthase activity was low: 9% for the first entrapment, and 2% for the second entrapment. Therefore, each entrapment step lost 80% of enzyme activity. This may be indicative of

TABLE III

10 Purification of Cellulose Synthase from Acetobacter  
xylinum

Activity	Specific Activity					
	Total Protein		nmol/min/mg <sup>#</sup>			
Recovery	Step	mg	%	Mean	Best	%
Washed membranes		396*	100	10		100
Triton extract (TSt)		65	16.4	36	45	59
Cellulose entrapment						
Step 1 (EP <sub>1</sub> )		0.85	0.2	420	630	9
Step 2 (EP <sub>2</sub> )		0.085	0.02	950	1560	2

\*Values were derived from 8 gm (dry weight) of cells.

#Mean specific activity represents the values from 5 sets of purifications. Best specific activity represents the value for the individual set of highest specific activity.

35 Gel Electrophoresis of the Purified Enzyme - The enzyme  
preparations from each purification step were analyzed by  
LDS-PAGE. In contrast to the protein bands from TS (Fig.  
2, lane 1), very few of the protein bands over 66 Kd were  
observed from TSt (Fig. 2, lane 2). For the purified  
40 preparations from the trypsinized washed membranes, two  
major bands, 93 Kd and 83 Kd, were found in the gel from  
the EP<sub>1</sub> or EP<sub>2</sub> fraction (Fig. 2, lane 4 and 5).  
Overloading of EP<sub>2</sub> in the gel (Fig. 2, lane 5) produced 93  
Kd and 83 Kd bands which accounted for 90% of the total  
45 density of stain in the gel lane. These two protein bands  
also were seen in the EP<sub>1</sub> fraction purified from TS, which

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was prepared from the trypsin-untreated washed membranes (Fig. 2, lane 3).

5 In addition to two major protein bands, silver  
staining indicated some minor protein bands in the gel  
from the TSt-EP<sub>1</sub> fraction (Fig. 2, lane 6). For the most  
purified fraction, TSt-EP<sub>2</sub>, minor protein bands were still  
present in the gel (Fig. 2, lane 7 and 8). The 93 and 83  
kd polypeptides also accounted for about 90% of the total  
10 stain density.

15 By using n-octyl glucoside or digitonin, and following a similar purification procedure, the EP<sub>1</sub> fractions also produced 93 Kd and 83 Kd bands as the major components (Fig. 3, lane 1, 2 and 3). Analysis of the protein composition in the EP<sub>1</sub> fraction from the DS with and without a cushion solution (Fig. 3, lane 3 and 4) revealed that more protein contaminants were removed in the presence of the cushion solution.

Without a reducing agent treatment, a 101 Kd band appeared instead of the 93 Kd band (Fig. 4, lane 1 and 2). Two small protein spots remained at the 93 Kd position (Fig. 4, lane 1, arrowheads). The 83 Kd band exhibited no change in mobility (Fig. 4, lane 1 and 2).

Trypsin Effect on the Enzymes - Incubation of either the washed membranes or TS with trypsin did not effect enzyme activity after 30 minutes at 25°C. Also, if the trypsin was added to the EP<sub>1</sub> fraction from DS, enzyme activity was still maintained after 1 hour at 25°C; however, only the 83 Kd polypeptide was still present in LDS-PAGE while the other polypeptides were degraded after 10 minutes incubation at 25°C (Fig. 4, lane 3, 4 and 5).

35

Characterization of the Glycosylated Protein - The binding of cellulose synthase to Con A-Sepharose was examined. The TS was applied and passed through the column. After washing with buffer, the cellulose synthase was bound with Con A. It could be easily eluted by addition of the enzyme buffer with 0.3 M methyl  $\alpha$ -D-mannopyranoside (Fig.

17% of the protein was recovered, and a 12-fold purification was achieved by this method.

10

When the purified EP<sub>1</sub> fraction from DS was separated by LDS-PAGE in the absence of reducing agents, and then stained using FITC-Con A, three bands of 110 Kd, 103 Kd and 83 Kd were found to react with the stain (Fig. 6, lane 2). The 83 Kd polypeptide fluoresced much more strongly than the other two bands. This band also fluoresced more strongly when stained with the Schiff-periodate method using dansyl hydrazine (Fig. 6, lane 1).

20

Gel Filtration Chromatography - The Triton-solubilized enzyme preparation, TS, was applied to a Sephadryl S-300 column for determination of the molecular weight of native cellulose synthase. The elution profile of cellulose synthase was determined by measuring the enzyme activity in the column fractions (Fig. 7A). There were two peaks of enzyme activity in the elution profiles. By the use of molecular weight markers as standards of Stokes' radii, the molecular weights of 490 Kd and 360 Kd for TS were shown to correspond to the two peaks of activity (Fig. 7A). The range was between 650 Kd to 300 Kd in TS.

30

Therefore, the Stokes' radius of this enzyme was shown to be about 5.5-7.5 nm. After treatment with trypsin at 25°C for 10 minutes, the TS was applied to the column. The molecular weight range of the cellulose synthase was broadened to between 650 Kd and 100 Kd, and two peaks were

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observed to correspond with the molecular weights of 490 Kd and 300 Kd (Fig. 7B).

After gel filtration, the recovery of cellulose activity was about 60% of total enzyme activity in the originally applied Triton-solubilized preparation (TS). For the trypsin-treated TS, there was no loss of enzyme activity as compared with total activity of the original sample. In addition, the specific activity of the peak fraction from trypsin treated sample was approximately twice as large as the nontreated sample (Fig. 7A and 7B).

**EXAMPLE II: IDENTIFICATION OF THE ACTIVE SUBUNIT OF CELLULOSE SYNTHASE**

Photoaffinity labeling - [ $\beta$ -<sup>32</sup>P]5N<sub>3</sub>UDP-glc was synthesized by a procedure described by Drake et al. (1989). In addition to 20  $\mu$ l of activator for cellulose synthase, each reaction mixture contained 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 1mM EDTA, the indicated protein quantity of enzyme sample, and 20  $\mu$ M of [ $\beta$ -<sup>32</sup>P]5N<sub>3</sub>UDP-glc (specific activity, 24 mCi/ $\mu$ mol) in a final reaction volume of 50  $\mu$ l at pH 8.0-8.5. For competition experiments, the competitor, namely UDP-glc, was added at final concentrations of 0, 50, 100, and 300  $\mu$ M, respectively. For saturation studies, [ $\beta$ -<sup>32</sup>P]5N<sub>3</sub>UDP-glc was added at final concentrations of 20, 50, 100, 300 and 500  $\mu$ M, respectively. Reactions were incubated for at 30 degrees centigrade for 30 seconds, followed by short wavelength UV irradiation (254 nm) with a hand-held UV lamp (Model UVSL-58, Ultraviolet Products, Inc.) in open Eppendorf microcentrifuge tubes at a distance of 4 cm for 60 sec. Reactions were terminated by the addition of 0.3 ml of 7% perchloric acid. After 20 minutes, the samples were centrifuged for 3 minutes in an Eppendorf model 5412 centrifuge. The pellet was resuspended in LDS sample

buffer for subsequent LDS-PAGE as previously described (Lin and Brown 1989). The Coomassie blue stained gel was dried and autoradiographed. Autoradiography was performed at -80°C with Kodak X-Omat AR film and a Dupont Cronex intensifying screen. The molecular mass standards (Bio-Rad) were phosphorylase b (97Kd), bovine serum albumin (66

10 The quantitation of the autoradiographs was measured by grey value discrimination of a digitized image (recorded on a Kontron/Zeiss IBAS Image Processing System). From this data, the corresponding molecular weight was calculated.

15 Results of Purification and Photoaffinity Labeling Studies  
- Two crude enzyme preparations, membranous and digitonin-solubilized forms, and the purified enzyme were tested for their ability to photoincorporate [ $\beta$ -<sup>32</sup>P]5N,UDP-glc (Fig. 8). With or without the presence of c-di-GMP, 20 there was no labeling of the 83 Kd band found in the membrane fraction except for the 57 Kd protein band (Fig. 8, lanes 1 and 2). A similar labeling of a 57 Kd band was observed in the digitonin-solubilized enzyme preparation (Fig. 8, lanes 3 to 4); however, a faintly photolabeled 25 band corresponding to the 83 Kd polypeptide was seen in the presence of c-di-GMP (Fig. 8, lanes 2 and 4). In the purified preparation, the 83 Kd band became intensely labeled after azido UDP-Glu photolabeling (Fig. 8, lane 5) and the photoincorporation increased 45% more in the 30 presence of c-di-GMP (Fig. 8, lane 6).

Figure 9 shows a Coomassie blue stained gel with the corresponding autoradiograph, demonstrating photolabeling of the cellulose synthase in various fractions purified 35 using different detergents. In all cases, the 83 Kd protein band was exclusively photolabeled with [ $\beta$ -

<sup>32</sup>P]5N<sub>3</sub>UDP-glucose (Fig. 9B, lanes 2-4). Control experiments in which the reaction mixtures were not irradiated with UV light (Fig. 9A and 9B, lane 1) or in which pre-irradiated [ $\beta$ -<sup>32</sup>P]5N<sub>3</sub>UDP-glucose was used (Fig. 11, lane 1), yielded no labeling of the 83 Kd polypeptide or any other polypeptides.

10 UDP-glucose was known to be the glucosyl donor for cellulose synthase and was selected as a competitor against photoinsertion of  $[\beta-^{32}\text{P}]5\text{N}_3\text{UDP-glucose}$ . As competitor concentrations increased from 50 to 300  $\mu\text{M}$  UDP-glc, the photoinsertion of the probe into the 83 Kd polypeptide became reduced (Fig. 10), with a  $K_d$  of 82  $\mu\text{M}$ . These UDP-glc protection studies provided further evidence 15 to validate the specificity of the photoaffinity labeling.

A test of a probe's utility for active site studies has been shown to be the ability to saturate the available binding sites. Figure 11 shows the saturation of cellulose synthase photolabeling by  $[\beta-^{32}P]5N_3\text{UDP-glc}$ , with a  $K_d$  value of 68  $\mu\text{M}$ . The photoinsertion of the probe into the 83  $\text{Kd}$  protein band showed that saturation was being obtained with  $[\beta-^{32}P]5N_3\text{UDP-glc}$  at concentrations just above 100  $\mu\text{M}$ . This demonstrated that the photoprobe was binding to a limited number of sites and that nonspecific photoinsertion under these conditions was minimal.

The 57 Kd band in the digitonin-extracted enzyme preparations was labeled either by using preirradiated [ $\beta$ -<sup>32</sup>P]5N<sub>3</sub>UDP-glc solutions (Fig. 12, lane 1) or in the absence of photoactivation by UV irradiation (Fig. 12, lane 2), as well as by normal photolysis (Fig. 12, lane 3). These results indicated that the 57 Kd protein was not interacting with the photoprobe and that labeling was independent of a photoactive precursor. Furthermore, the labeling of this protein was inhibited in the presence of

500  $\mu$ M glc-1-P or glc-6-P (Fig. 12, lanes 4 and 5). It was not inhibited in the presence of glc-1,6-P (Fig. 12, lane 6) a fact consistent with the properties of phosphoglucomutase [Drake (1989); Ray and Peck (1972)].

5

There are three major requirements for demonstrating

10 studies: (a) only a specific protein(s) in an enzyme preparation is photolabeled with the photoprobe; (b) specific photoincorporation at the active site is measured by the ability of the probe to saturate the binding sites and by prevention of photolabeling with the native substrate at appropriate concentrations; and (c) the photolabeling is dependent on the presence of activating 15 light to exclude pseudo-photoaffinity labeling.

20 The present inventor's results with the purified cellulose synthase preparation met all of these criteria. Thus, these studies confirmed the earlier suggestion (Lin and Brown 1989) and provided solid evidence that the 83 Kd polypeptide was the catalytic subunit of the cellulose synthase from Acetobacter xylinum strain ATCC 53582. Further strong evidence was that c-di-GMP substantially 25 increased the photoinsertion of [ $\beta$ -<sup>32</sup>P]5N,UDP-glc with the 83 Kd protein. This cyclic nucleotide has been shown to be a true activator for cellulose synthase in A. xylinum (Ross 1987). The fibrillar structure and cellulosic 30 nature of in vitro product has been visualized and characterized by electron microscopy (Lin 1985) Using X-ray diffraction analysis, the in vitro product from purified enzyme preparation or membrane fraction (Bureau and Brown 1987) was identified as cellulose II.

35 In the presence of the cellulose synthase activator, a weakly photolabeled 83 Kd polypeptide in the membrane fraction or the digitonin-solubilized preparation was

still detectable (Fig. 8, lanes 2 and 4). In contrast, no detectable labeling was observed when no activator was added (Fig. 8, lanes 1 and 3). Again, this demonstrated that c-di-GMP, the specific activator of cellulose synthase, truly increased the affinity of cellulose synthase for UDP-glc binding and produced a detectable photoactivated labeling of  $[\beta-^{32}P]5N_3$ UDP-glc. Two possible reasons for the weak photolabeling of the 83 Kd protein band from these enzyme preparations are as follows: (a) these crude preparations contain trace amounts of the cellulose synthase and therefore would be expected not to label as heavily as the purified enzyme preparation, and (b) the efficiency of photoinsertion as well as protein concentration affects this labeling.

The 57 Kd protein band labeled in the crude membrane fraction and the digitonin-solubilized enzyme preparation is close to the molecular weight previously reported for phosphoglucomutase in the Gram-negative bacteria (Ray and Peck 1972). Though phosphoglucomutase was reported as a soluble enzyme, labeling of a 62-64 Kd protein following incubation with  $[\beta-^{32}P]5N_3$ UDP-glc has been observed in several membrane preparations (Drake and Haley, unpublished). This labeling has been attributed to trace levels of  $[\beta-^{32}P]glc-1-P$  present in the photoprobe preparations or due to enzymatic hydrolysis of the photoprobe. Whether the 57 Kd protein was a membrane-bound isozyme of phosphoglucomutase or was an artifact of the membrane isolation technique was not determined. These experiments confirmed that the 57 Kd protein was a phosphoglucomutase.

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**EXAMPLE III: USE OF THE AZIDO-UDP-GLUCOSE PHOTOPROBES TO  
IDENTIFY GLUCAN SYNTHASES IN VASCULAR PLANTS**

5       Azido-UDP-Glc photolabeling of cotton fiber plasma membranes (+ UV but without activator/s) revealed only a weak photoinsertion of a 66 Kd polypeptide band, and occasionally very faint bands.

      [REDACTED] INVENTORS TO ATTEMPT TO ENHANCE PHOTOLABELLING AS WAS SUCCESSFUL IN A. xylinum.

10       One approach which was attempted was similar to that attempted in A. xylinum. With activators from crude GX, an effector solution from Acetobacter which contains cyclic-di-GMP and other components, a significant increase 15 was achieved in azido-UDP-Glc photoinsertion (Figure 14; lanes 3, 4, and 5). A 66 Kd band is prominently photolabeled, and two other bands, one at 57 Kd and the other at 35 Kd band are photolabeled.

20       In addition, a specific agent, a heated deproteinized supernatant derived from a membrane fraction of Acetobacter (called "crude factor"), fraction X-1 which has a maximum absorbance at 228 nm, is heat stable, ethanol soluble, and sensitive to phosphodiesterase (lane 25 4). Azido-UDP-Glc photolabeling studies with the heated supernatant (lane 8) above showed an increase in photoinsertion of 5-fold for the 66 Kd band, 6-fold for the 57 Kd band, and 8-fold for the 35 Kd band. Pure cyclic-di-GMP, the specific allosteric activator for 30 cellulose synthase in Acetobacter had no effect on azido-UDP-Glu photolabeling of the 66, 57, or 35 Kd bands (lane 9).

35       Certain factors affect the intensity of the photolabeling of glucan synthase polypeptides. Azido-UDP-Glc photolabeling of these polypeptides is most intense in

preparations derived from fibers which are 30 days post anthesis. This period corresponds to the period of greatly increased secondary wall formation of virtually pure cellulose. Labeling of two of these proteins (35 Kd and 57 Kd) were abolished in dark controls (without UV irradiation, but with activator (lane 1)) and the 66 Kd band was weakly labeled under these conditions.

Photolabeling of all three polypeptides was abolished in the presence of 1 mM unlabeled UDP-Glc, thus establishing specificity of azido-UDP-Glc photoaffinity binding (see Lin, et al, 1990 for comparable details).

Experiments with azido-UDP-Glc revealed that saturation of azido-UDP-Glc by photoinsertion was obtained at a concentration of approximately 100  $\mu$ M. The three photolabeled bands had differing apparent Kd's of 15  $\mu$ M for the 66 Kd band, 22  $\mu$ M for the 35 Kd band, and 25  $\mu$ M for the 57 Kd band.

All three polypeptides require either Ca<sup>++</sup> or Mg<sup>++</sup> to effectively photolabel. High Ca<sup>++</sup> concentration coupled with low Mg<sup>++</sup> or vice versa, do not significantly change azido-UDP-Glc photolabeling. However, when Mg<sup>++</sup> is replaced by Mn<sup>++</sup>, photolabeling of the 35 Kd band is completely abolished while the 66 Kd band is greatly reduced. On the other hand, when Mg<sup>++</sup> is replaced by Zn<sup>++</sup>, the photolabeling of the two bands does not change. The 66 Kd, 57 Kd, and 35 Kd bands are believed to represent at least two different glucan synthases.

**EXAMPLE IV: CLONING AND SEQUENCING OF THE GENE FOR THE CATALYTIC SUBUNIT OF CELLULOSE SYNTHASE FROM ACETOBACTER XYLINUM**

The purification of cellulose synthase activity and identification of the 83 Kd polypeptide by LDS-PAGE to be the catalytic subunit of cellulose synthase provided the

present inventors the necessary tools for attempting to clone the DNA encoding this important enzyme. N-terminal amino acid sequencing of the 83 Kd polypeptide was carried out as follows. Samples of the highly purified protein 5 were loaded onto gels containing 8% polyacrylamide and electrophoresed according to Lin and Brown (1989). After

transferred to a polyvinylidene difluoride (PVDF) membrane (Matsudaria 1987). The 83-Kd polypeptide band was cut out 10 with a clean razor. The protein from the excised bands were sequenced using an Applied Biosystems model 477A pulse liquid phase sequenator (carried out at the Department of Zoology at the University of Texas at Austin, Texas).

15 The amino acid sequence of the 83 Kd polypeptide was initially attempted on the purified cellulose synthase of the present invention without being treated with trypsin but this approach resulted in ambiguities at a number of 20 positions during the sequencing and was not used in the design of the oligonucleotide probes. The initial sequence obtained is shown below: (Amino acid residues believed to be ambiguous are italicized.)

25 Met-Glu-Val-Arg-Gly/Ser-Leu/unknown-Thr-Gln-Leu-Arg-Leu-Gly-Asn-Thr-Leu-

30 While not of use for designing probes, the sequence above was useful in localizing the coding sequence eventually obtained.

The amino acid sequence of the 83 Kd polypeptide from purified cellulose synthase was obtained from trypsin-treated membrane preparations as described earlier herein. 35 Specifically, trypsinized membranes were prepared as follows: to 10 ml of washed membranes (protein

concentration, 5 mg/ml), 0.2 mg of trypsin was added and gently shaken at 25°C for 30 minutes. After the addition of 0.2 mg of trypsin inhibitor for 20 minutes, the mixture was centrifuged at 18,000 xg for 20 minutes and washed 5 once in TME.

The trypsinized membranes were resuspended in TME containing 20% glycerol, 0.6% (wt/vol) Triton X-100 to give a final protein concentration of 5.0 mg of membrane 10 protein per ml. After homogenization with a teflon homogenizer, the suspension was gently shaken for one hour, followed by centrifugation at 100,000 xg for one hour. The supernatant represents the Triton-solubilized 15 enzyme which is designated "TSt" since the trypsinized membranes are used.

The entrapment method used here was similar to that used previously (Lin and Brown 1989) and is described as follows: cellulose nitrate tubes, 9/16 x 3 3/4 in., were 20 used. 2.6 ml of cushion solutions, consisting of TME buffer and 30% glycerol (v/v), were added to the bottom of the tubes. Subsequently, a mixture of 6.8 ml of the solubilized trypsin-treated enzyme, 0.2 ml of 100 mM UDP-glucose and 3.4 ml of N-Gx was layered on the top of 25 cushion solution. The tubes were incubated in a 30°C water bath for 11 minutes then were placed on ice for two hours, and centrifuged for 20 minutes in a Beckman SW 40 Ti rotor at 50,000 xg. After overnight storage at -80°C, each pellet was resuspended in extraction buffer to 1/30 30 the original loading volume and homogenized with a Teflon homogenizer. (Extraction buffer was prepared with 0.1% of detergent in TME buffer containing 20% glycerol). The suspension was centrifuged for 10 minutes at 15,000 xg. The 35 supernatant was designated ES1. The EP1 was resuspended in extraction buffer to the same volume as ES1. The purified protein was then used for protein

sequencing studies as described above. The resulting trypsinized peptide sequence is shown below:

**Ser-Ser-Thr-Gln-Ser-Glu-Ser-Gly-Met-Ser-Gln-Trp/Leu-Met-Gly-Lys**

5

10 amino acid sequence of Fig. 1, the resulting sequence was fortuitously close to the amino terminal end of the cellulose synthase (amino acid residues 6-20). Once an amino acid sequence was determined with a sufficiently low level of ambiguity, design and synthesis of oligonucleotide (17-mers) from the amino acid sequence data was undertaken for use as probes in DNA-DNA hybridization. For designing oligonucleotide probes from the amino acid sequence, the inventors selected a stretch of six amino acids (-Met-Ser-Gln-Trp/Leu-Met-Gly-) from the trypsin treated 83 Kd polypeptide sequence. Eight 17-mer oligonucleotides, each with a unique sequence and having deoxyinosine (I) by the ambiguous position of the serine codons, were synthesized at Operon Technologies, Inc., California. The sequences of the oligonucleotide so constructed are given below:

15

20

25	83-1A	5'-ATGTCICAATGGATGGG-3'
	83-1G	5'-ATGTCICAGTGGATGGG-3'
	83-2A	5'-ATGAGICAATGGATGGG-3'
	83-2G	5'-ATGAGICAGTGGATGGG-3'
	83-3A	5'-ATGTCICAACGTGATGGG-3'
30	83-3G	5'-ATGTCICAGCTGATGGG-3'
	83-4A	5'-ATGAGICAACGTGATGGG-3'
	83-4G	5'-ATGAGICAGCTGATGGG-3'

35 Hybridization of the oligonucleotide probes with A. xylinum DNA was carried out using the eight oligonucleotides that were thus synthesized. The probe

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that showed maximum homology with DNA from A. xylinum ATCC 53582 was determined by DNA-DNA hybridization. In order to accomplish these studies, total DNA from A. xylinum ATCC 53582, A. xylinum ATCC 23769, and E. coli HB101 (a common strain of E. coli used in recombinant DNA experiments) was digested with HindIII and the fragments were separated on an agarose gel. After separation, the fragments were transferred by capillary action to a nylon membrane (GeneScreen; NEN Research Products) according to the procedure of Reed and Mann (1985). The DNA thus transferred was next hybridized with the <sup>32</sup>P-labeled oligonucleotide of the invention using standard procedures. Hybridization was accomplished in a solution with 5X SSC [1X SSC: 0.15M NaCl, 0.015M sodium citrate] at 45°C for 16-24 hours. The filters were then washed with a solution containing reduced salt concentration. In all cases, the hybridization temperature was 5-7°C below the dissociation temperature as described by Suggs, et al. (1981) applying the formula  $T_d = 2(A+T) + 4(G+C)$ .

20

Oligonucleotide 83-1G showed homology with a single HindIII fragment from A. xylinum strains ATCC 53582 and ATCC 23769 under stringent hybridization conditions during which none of the other oligonucleotides exhibited significant homology. To the surprise of the present inventors, all eight oligonucleotides showed hybridization to DNA from E. coli HB101 even under stringent conditions (at least 5 HindIII fragments hybridized). This fact prevented the use of standard procedures utilizing recombinant DNA libraries in E. coli.

30  
That the 83-1G oligonucleotide was hybridizing with a unique DNA sequence from A. xylinum was confirmed by hybridizations with DNA digested with NruI, NarI, SmaI, ClaI, and BglII. In all cases, hybridization was observed 35 with a single fragment of A. xylinum DNA.

Once the hybridization studies had been accomplished, cloning of the A. xylinum ATCC 53582 DNA fragment homologous to oligonucleotide 83-1G was carried out. The HindIII fragment of A. xylinum ATCC 53582 (~9.5 kb) showing homology to 83-1G oligonucleotide was cloned following isolation of A. xylinum DNA fragments in that

HindIII-cleaved pUC18. DNA fragments were isolated from low melting temperature agarose using the elution tip technique. The fragments were ligated with HindIII cleaved pUC18 using T4 DNA ligase (BRL) at 15°C for 8 hours. The ligation mixture was transformed into E. coli DH5-alpha where colonies carrying recombinant plasmids could be screened for the absence of  $\beta$ -galactosidase activity on medium with X-gal. Very few recombinants were obtained in this strain, possibly due to restriction of A. xylinum DNA in the Mcr+ background of this strain, and none of the recombinant plasmids carried the fragment homologous to 83-1G.

Therefore, the ligation mixture was next transformed into E. coli HB101 (a recA strain) and transformed colonies were selected on medium containing ampicillin (the selectable marker on pUC18). As pointed out earlier, this strain of E. coli made it difficult to screen colonies for recombinant plasmids since there was strong hybridization of the probes with the DNA of these cells. Therefore, plasmid DNA was isolated from pooled collections containing 50 Amp<sup>r</sup> colonies each and was analyzed by hybridization with <sup>32</sup>P-labelled 83-1G as probe. This screening resulted in the identification of the plasmid pIS532 that carried a HindIII fragment of 9.5 Kb from A. xylinum ATCC 53582 homologous to 83-1G.

Sequencing of the A. xylinum cellulose synthase catalytic subunit gene was accomplished by first

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constructing a restriction map of pIS532 using various restriction enzymes. An EcoRI fragment of ~950 bp was identified by hybridization to carry the DNA sequence homologous to 83-1G oligonucleotide. This fragment was 5 cloned into the vector M13mp19 in both orientations and DNA sequencing was performed by the dideoxy chain-termination method. The sequence of the 83-1G oligonucleotide was identified from the sequence obtained and the reading frame was established from the known amino 10 acid sequence of the 83 Kd polypeptide. Cloning and sequencing of other fragments from pIS532 was done using M13mp18 and M13mp19 vectors. An analysis of the sequence obtained led to the identification of an open reading frame of 2169 bp coding for a polypeptide of approximately 15 80 Kd. The first 20 amino acids deduced from the DNA sequence matched those obtained for the approximately 83 Kd polypeptide corresponding to the purified cellulose synthase subunit.

20 In summary, the DNA sequence for the catalytic- subunit of cellulose synthase from A. xylinum ATCC 53582 was identified using oligonucleotide probes designed from the amino acid sequence of an 83 Kd polypeptide that was obtained following purification of the cellulose synthase 25 activity outlined in the examples above. A 9.5 kb HindIII fragment of A. xylinum DNA carrying the gene for the cellulose synthase catalytic-subunit was cloned in the vector pUC18. DNA sequencing was performed by the dideoxy chain-termination method following cloning of smaller 30 fragments in M13mp18 and M13mp19. An open reading frame of 2169 bp coding for a polypeptide of 80 Kd was identified from the sequencing data in which the first 20 amino acids matched with the N-terminal amino acid sequence of the 83 Kd polypeptide.

**EXAMPLE V: CLONING AND SEQUENCING OF THE GENE ASSOCIATED WITH THE CATALYTIC SUBUNIT OF CELLULOSE SYNTHASE FROM ACETOBACTER XYLINUM**

5           *A. xylinum* strain ATCC 53582 was used for  
purification of the cellulose synthase and isolation of  
the 83 Kd polypeptide.

10 gene for the 93 Kd polypeptide has been described above. N-terminal amino acid sequence of the 93 Kd polypeptide was determined in the same manner as described for the cellulose synthase catalytic subunit.

For DNA sequencing of the 93 Kd polypeptide coding region, fragments of pIS532 were cloned in M13mp 18 and M13mp19 in both orientations and deletion derivatives were prepared according to the method of Henikoff using the Erase-a-Base kit from Promega, Madison, WI. Sequencing was done by the dideoxy chain-termination procedure using the Sequenase version 2.0 kit from U.S. Biochemical Corp., Cleveland, OH. DNA and amino acid sequences were analyzed using the PC/GENE programs and the Intelligenetics suite.

## Identification of the Gene for the 93 Kd Polypeptide From the N-Terminal Amino Acid Sequence

DNA sequence beyond the stop codon of the cellulose synthase catalytic subunit gene, from plasmid pIS532, showed no transcription termination signal. Instead, an open reading frame with an ATG start codon was observed at 5 position 2860, suggesting the presence of other gene(s) downstream of the catalytic subunit gene. Sequencing of the downstream region led to the identification of an open reading frame starting at position 2860 and terminating at position 5289, coding for a polypeptide of MW 86 Kd (Fig. 10 13). A potential ribosome-binding site with the sequence GGAACC is present 6 base pairs upstream of the putative start codon. The deduced amino acid sequence of this polypeptide from amino acid position 27 to 40 is found to be identical to the sequence of the first 14 N-terminal 15 amino acids determined for the 93 Kd polypeptide that is shown here:

20 Ala-Pro-Ala-Trp/His-Gln-Pro-Glu-Ala-Thr/Ser-Asp-Leu-Pro-Pro-Leu-Arg/Leu.

25 The amino acid sequence identity between that determined for the 93 Kd polypeptide and one deduced from the DNA sequence confirms that this sequence codes for the 93 Kd polypeptide.

30 25 No N-terminal methionine is observed in the amino acid sequence determined for the 93 Kd polypeptide. The N-terminal sequence obtained could result from either cleavage of a signal peptide or from cleavage of a N-terminal fragment by trypsin used in the purification of the cellulose synthase. No N-terminal sequencing of the polypeptide which was purified from non-trypsin treated membranes was accomplished to determine if trypsin had cleaved an N-terminal fragment as was shown for the 35 cellulose synthase catalytic subunit.

The amino acid sequence derived from the DNA sequence shows occurrence of a number of potential signal peptide cleavage sites. Potential cleavage sites at the N terminal end of the polypeptide are present at positions 14,15; 16,17; 18,19; 19,20; and 24,25. The cleavage site at position 16-17 shown in the sequence is the most likely and could be the actual cleavage site for the signal peptide.

10 The amino acid sequence at the N-terminal end of the processed polypeptide still differs from the amino acid sequence determined for the 93 Kd polypeptide, suggesting that a few more amino acids were removed following cleavage of the signal peptide. Since the 93 Kd 15 polypeptide was purified from trypsin treated membranes, a short fragment of the processed polypeptide appears to be cleaved by trypsin at the N-terminal end. This is suggested by the amino acid sequence obtained for the 93 Kd polypeptide which is found to start after lysine at 20 position 27 in the deduced amino acid sequence.

Even though the nucleotide sequences that are disclosed herein for the cellulose synthase catalytic subunit gene and the 93 Kd polypeptide gene are homologous to the sequences of the *bcsA* and *bcsB* genes respectively [Wong et al. 1990], they are not identical. This divergence could be due to the different strains of *A. xylinum* used in the two studies. Variability for cellulose production is commonly observed between different strains of *A. xylinum*, and this variability might very well be due to the differences in the DNA sequences coding for the various polypeptides involved in cellulose synthesis. Southern hybridization of DNA coding for the cellulose synthase catalytic subunit from *A. xylinum* ATCC 53582 with DNA from other strains of *A. xylinum* shows homology, but the size of DNA fragments

5 showing hybridization and the intensity of the hybridization signal is found to vary with the strains suggesting that there is a difference in the DNA sequences of these strains. Whereas some strains of A. xylinum seem closely related to one another, other strains are distantly related. Such variation of nucleotide sequences between different strains of bacteria is commonly observed, as in the case of the *trp* region in E. coli.

10 From the data disclosed herein, it was conclusively shown that the 83 Kd polypeptide, obtained from purified cellulose synthase and identified by photolabelling with [<sup>32</sup>P]5-azido UDP-glucose is the cellulose synthase catalytic subunit. Moreover antibodies raised against the 15 93 Kd polypeptide, when used in cellulose synthase enzyme assays, showed no effect on enzyme activity suggesting that the 93 Kd polypeptide was not the catalytic subunit.

20 Whether the cellulose synthase catalytic subunit can bind UDP-glucose on its own or requires some other polypeptides for the binding is not known at present. However, it is known that the activator c-di-GMP enhances the binding of UDP-glucose to the catalytic subunit suggesting that the activator-binding site is either 25 present on the catalytic subunit or on another polypeptide that is tightly associated with it.

30 The present inventors propose that the cellulose synthase catalytic subunit and the 93 Kd polypeptide are tightly associated and are involved in the polymerization reaction. Other polypeptide(s), that probably are coded for by gene(s) in the cellulose synthesizing operon, may be loosely associated with the cellulose synthase complex 35 and could therefore not be copurified with the cellulose synthase activity. These polypeptides may be involved in the crystallization process either directly by organizing

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the glucan chains for efficient, directed export or  
indirectly by organizing the various polypeptides into a  
pore-like structure through which the glucan chain  
aggregates are guided facilitating the assembly of  
5 cellulose I microfibrils and ribbons.

**VI. TRANSFORMATION OF COTTON WITH  
RECOMBINANT B-1,4 GLUCAN SYNTHASE**

10 The following example follows the method of  
Firoozabady (1987). It is proposed that this method may  
be used in combination with the present invention for  
transforming cotton with a recombinant glucan synthase  
gene and for obtaining transgenic plants therefrom. As a  
15 control for efficacy of the method when applied to glucan  
synthase, it is proposed that one may duplicate as closely  
as possible the method described below and make only those  
changes as are required from the inherent differences  
between the bacterial antibiotic resistance gene and the  
20 cellulose synthase gene.

It will be obvious to those of skill in the art that  
cotton and other plant species already possess functional  
glucan synthases. It is proposed here that certain  
25 utilities and advantages may be realized in improving the  
target host plant. This may be accomplished by  
introducing a modified (such as by site-directed  
mutagenesis or by construction of fusion proteins or by  
coupling with alternative promoter systems) homologous  
30 gene from the same plant species itself back into the same  
plant's cells. For instance, such a transformation might  
be accomplished by reintroducing the recombinant cellulose  
synthase gene derived from cotton by the methods and  
compositions of the present invention back into cotton  
35 cells but which gene was modified by placing the gene  
under the control of different promoters which then  
express the recombinant gene. Alternatively, the

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transformations described here may be accomplished using recombinant cellulose synthase-encoding fragments from sources other than the host cell.

5        Bacterial Strains and Plasmids

10        Escherichia coli strains MC1061 (Casadaban and Cohen 1980) or K802 (Wood 1966) are employed in the recombinant DNA procedures. MM294(pRK2013) is employed as a plasmid-mobilizing strain (Ruvkun and Ausubel 1981) in triparental matings and 2174 (pPH1J1) as an excluding plasmid donor in marker exchange experiments (Garfinkel 1981). A.  
15        tumefaciens strains include 15955 (American Type Culture Collection) and LBA4404 (Hoekema 1983). E. coli strains are grown in L medium (Maniatis 1982) at 37°C and A.  
20        tumefaciens strains in YEP (1% yeast extract, 1% peptone, 0.5% NaCl) or Schilperoort's minimal (SM) medium (Klapwijk 1975) with 0.2% sucrose, at 28°C. Antibiotics (mg/l) are used for selecting E. coli and include: ampicillin, 50; kanamycin, 25; tetracycline, 10; gentamycin, 10. The latter three antibiotics are typically used for  
25        Agrobacterium selection at 25, 10, and 100mg/l, respectively and streptomycin at 250 mg/l. Plasmids PRK292 (Ditta 1985) and pTJS75 may be obtained from the laboratory of D. Helinski (University of California, San Diego).

Binary Vector System

30        The details of the construction of the binary vector pH575 are available elsewhere (Sutton 1987). The cloning and recombinant DNA manipulations (Maniatis 1982) of pH575 and its derivatives are all performed using E. coli MC1061 as the host strain. Because this strain is resistant to  
35        streptomycin, it is unsuitable for use as a donor strain for conjugations into LBA4404. Consequently, the binary

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vector is transformed into *E. coli* strain K802 for use in triparental matings (Kemp 1983).

5 For the control experiments, streptomycin- and kanamycin-resistant colonies are purified, and transconjugants are identified by a ~~bioassay~~ ~~bioassay~~ lysis procedure (Maniatis 1982).

10 The DNA between the border repeats of the transposon carrying the kanamycin gene is mobilized in *trans* for integration into plant cells by *vir* gene functions borne on the co-resident Ti plasmid pAL4404. For the purposes of integrating cellulose synthase into the plant cells, a suitable fragment of the cellulose synthase containing plasmid of Example III above (pIS532) is used to replace 15 the kanamycin gene in pH575 while maintaining the transposable characteristics of the end repeats. This plasmid is designated pH575-cellulose synthase.

#### Cis Vector System

20 As a control line of experiments, a *cis* vector system based on *A. tumefaciens* 15955 is constructed by using the T-region from pH575 to replace the wild-type Ti sequences of pTl15955. The 9530-bp HindIII-partial fragment of 25 pH575 that contains the T-region is re-cloned into the unique HindIII site of PRK292. The resulting plasmid, pH592, is then mated from K802 into a streptomycin-resistant spontaneous mutant of wild type 15955, and transconjugants 15955(pH592) are selected for resistance 30 to streptomycin, kanamycin, and tetracycline on SM medium. Single crossover events, by which a cointegrate plasmid containing pH592 and pTl15955 sequences is generated, is selected on SM by resistance to kanamycin, tetracycline and gentamycin following a second triparental mating in 35 which plasmid pPH1J1 is introduced into 15955(pH592). At least one of these colonies is then subjected to five

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rounds of cycloserine enrichment as described (Koziel 1983), and at least approximately 2150 single colonies are screened for sensitivity to tetracycline. At least one colony is selected, and the expected structure of the T-DNA, resulting from a second crossover event that eliminates both the pRK292 sequences and  $T_1$ -DNA bp 1016 to 11207 sequences (Barker 1983), is verified by DNA blot analysis.

10       A *cis* vector system based on *A. tumefaciens* 15955 is also constructed by using the T-region from pH575-cellulose synthase to replace the wild-type Ti sequences of pTi15955. The HindIII-partial fragment of pH575-cellulose synthase, or some other suitable fragment that 15 contains the T-region is re-cloned into the unique HindIII site, or some other suitable site, of pRK292. The resulting plasmid is then mated from K802 into a streptomycin-resistant spontaneous mutant of wild type 15955, and transconjugants 15955(pH592-cellulose synthase) 20 are selected for resistance to streptomycin and tetracycline on SM medium.

25       Since kanamycin selection is not possible with the cellulose synthase marker, an approach similar to that used in the present invention including reprobing pooled plasmid DNA derived from transconjugants is used (Example III above). Single crossover events, by which a cointegrate plasmid containing pH592-cellulose synthase and pTi15955 sequences are generated, is selected on SM by 30 resistance to tetracycline and gentamycin following a second triparental mating in which plasmid pPH1J1 is introduced into 15955(pH592-cellulose synthase). At least one of these colonies is then subjected to five rounds of cycloserine enrichment as described (Koziel 1983), and at 35 least approximately 2150 single colonies are screened for sensitivity to tetracycline. At least one colony is

selected, and the expected structure of the T-DNA, resulting from a second crossover event that eliminates both the pRK292 sequences and T<sub>1</sub>-DNA bp 1016 to 11207 sequences (Barker 1983), is verified by DNA blot analysis.

5

#### Plant Material

Seeds of G. hirsutum cv. Coker 201 are surface-sterilized as described (Firoozabady and DeBoer 1986) except that seeds are exposed to bleach for only 8-10 minutes, germinated on MSO medium [hormone-free MS medium (Murashige and Skoog 1962), solidified with 0.2% Gel-rite (Kelko) and incubated as described (Firoozabady and DeBoer 1986)].

15

#### Transformation and Selection

The bacteria used for inoculation of cotyledon segments are scraped off the agar medium and suspended to a concentration of  $\approx 10^8$  cells/ml in a cotton callus initiation liquid medium (G<sub>2</sub>) containing MS salts (Gibco), 100mg/l myo-inositol, 0.4mg/l thiamine HCl, 5 mg/l 6-( $\gamma$ , $\gamma$ -dimethylallylamino)-purine (2iP), 0.1 mg/l 6-( $\gamma$ , $\gamma$ -naphthaleneacetic acid (NAA) (all from Sigma), 3% (w/v) glucose, pH 5.9. Cotyledon pieces ( $\approx 0.5$  cm<sup>2</sup> surface area) from sterile 12-day-old seedlings are dipped in the A. tumefaciens suspension in petri dishes and gently shaken for a few seconds to ensure contact of all cotyledon edges with the bacterial cultures. The cotyledon pieces are then blotted dry and placed on Whatman No. 1 filter paper on callus initiation medium G<sub>2</sub> solidified with 0.2% Gel-rite. Inclusion of the filter paper is not necessary for transformation but is suggested to greatly reduced bacterial overgrowth on plant tissues. Tissues are incubated at 25°C with a 16-h photoperiod (90  $\mu$ E m<sup>-2</sup> s<sup>-1</sup>).

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After three days cocultivation, cotyledon pieces are transferred to petri plates (without the filter paper) containing the same medium supplemented with 500 mg/l carbenicillin (to control bacterial growth) and, for control experiments, 15-35 mg/l kanamycin sulfate (U.S. Biochemicals). Tissues are incubated at 30°C with a 16-h photoperiod ( $90 \mu\text{E m}^{-2} \text{s}^{-1}$ ). After 3-4 weeks, calli are excised from the original explants, transferred to fresh medium, for control experiments to fresh kanamycin-containing medium, and incubated under lower light intensity ( $10 \mu\text{E m}^{-2} \text{s}^{-1}$ ).

#### Regeneration of Transgenic Plants

After two to three weeks, calli are placed and maintained on embryogenic medium ( $G_2$  with no hormones) and for experimental controls calli are placed under kanamycin selection (25 mg/l). Mature somatic embryos (5-10 mm in length with cotyledons, hypocotyl, and radicle structures) are transferred to GRMgn medium, which is a lower ionic strength medium (Stewart and Hsu 1977) modified by addition of 0.1 mg/l gibberellic acid (Sigma, filter-sterilized), 0.01 mg/l NAA (Sigma) and 0.5% glucose instead of sucrose (with no antibiotics), and incubated at 30°C, 16-h photoperiod ( $90 \mu\text{E m}^{-2} \text{s}^{-1}$ ) to germinate and form plantlets. Plantlets are transferred to GA-7 Magenta cubes (Magenta Corp.) containing GRMgn medium and incubated at 25°C with a 16-h photoperiod ( $90 \mu\text{E m}^{-2} \text{s}^{-1}$ ). Plants with 3 to 5 leaves are transferred to a commercial peat moss/perlite mixture (Growing Mix No. 2; Fafard Ltd., Canada), maintained in a growth chamber at 25°C night, 30°C day, 16-h photoperiod ( $150 \mu\text{E m}^{-2} \text{s}^{-1}$ ) and watered and fertilized as needed. Plants are gradually hardened off, repotted in soil and moved to a greenhouse.

TLC Octopine Assay

To detect octopine in callus or shoot material (as an indication of successful transformation), 10-20 mg fresh weight of tissue is incubated overnight on MSO medium

Tissues are homogenized in microfuge tubes, centrifuged (15000 g. 2 min), and 6  $\mu$ l of the supernatants is spotted (2  $\mu$ l at a time) on Whatman K-5 silica gel plates (20 x 20 cm) 1 cm apart. The plate is placed upright in a thin-layer chromatography (TLC) developing tank containing 20 ml of TLC buffer (methanol:2-butanol:0.1 M sodium acetate (pH 4.6), 15:1:4). After one hour, plates are removed, air-dried, sprayed faintly (under a fume hood) with a 1:1 fresh mixture of 10% NaOH in 60% ethanol: 0.04% phenanthrenequinone (Aldrich Chemical Co.) in 100% ethanol, and immediately visualized under UV light (254 nm).

The octopine assay described herein is suggested to be highly reliable. With this method, background artifacts or endogenous opines produced by feeding tissues on arginine (Christou 1986) are not anticipated to be present on the TLC plates at the time of scoring. This is due to the use of TLC buffer in which arginine migrates much more slowly on the plate than opines. It is also important that phenanthrenequinone staining be very light, and that the plate be read immediately after staining under UV light. The intensity of octopine production may be expected to vary, with some plants showing much stronger octopine production than others. In general, the improved sensitivity of the assay is likely to enhance the probability of detecting successful transformation of cotton callus with r combinant cellulose synthase.

**NPT II and Cellulose Synthase Assays: Enzyme-linked Immunoassorbent assay (ELISA) and immunoblot**

5           NPT II (ncomycinphosphotransferase II, the enzyme which confers kanamycin resistance) is detected and quantified in cotton extracts by an ELISA assay. The ELISA is constructed with rabbit anti-NPT II immunoglobulin G (IgG), a biotin conjugate of this antibody (Guesdon 1986) and streptavidin alkaline 10 phosphatase conjugate (Bethesda Research Laboratories). Rabbit anti-NPT II antiserum is raised against NPT II purified from E. coli containing plasmid pKS4 (Murai 1983). Purified NPT II is used as the standard for calculating NPT II content in tissue extracts. Similarly, 15 rabbit anti-cellulose synthase antiserum is raised against the highly purified cellulose synthase derived from A. xylinum or from the other cellulose synthase polypeptides of the present invention.

20           Plant extracts for ELISA is made by grinding four 5-mm leaf discs in 200  $\mu$ l of phosphate-buffered saline (127 mM NaCl, 2.6 mM KC1, 8.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>) containing 0.05% Tween-20, and 1% polyvinyl pyrrolidone 40000 (Sigma). Extracts are centrifuged (15000 g, 5 min) 25 and three three-fold dilutions of the sample supernatants are loaded into ELISA plates. Soluble protein concentrations are determined by the Bradford (Bradford 1976) dye-binding assay (Biorad). NPT II levels are expressed as ng NPT II per mg of soluble protein in the 30 extracts, while a similar measurement and analysis is carried out with cellulose synthase.

35           Cotton leaf extracts for immunoblot analysis are prepared in 4% SDS, 5% 2-mercaptoethanol, 20% glycerol in 0.068 M Tris-HCl (pH 6.8), electrophoresed in 13% polyacrylamide gels (Laemmli 1970), and blotted to

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nitrocellulose. Blots are processed in either rabbit  
anti-NPT II IgG (0.1  $\mu$ g/ml) or rabbit anti-cellulose  
synthase followed by goat anti-rabbit IgG alkaline  
phosphatase conjugate (0.2  $\mu$ g/ml, Kirkegaard and Perry) by  
5 standard procedures (Towbin 1979). Radioimmune

the wash buffer and RIPA containing 3% BSA and 1% goat  
serum is used as the antibody dilution buffer. Blots are  
developed in nitroblue tetrazolium/5-bromo-4-chloro-3-  
10 idolyl phosphate (both from Sigma) substrate solution  
(Balke 1984).

#### DNA Isolation and Blot Hybridization

15 DNA is prepared by isolation of nuclei from cotton  
leaf tissues using a modification of the method of Murray  
and Kennard (Murray and Kennard 1984). Young leaf tissues  
are ground in a Bellico glass-glass homogenizer at 4°C in  
nuclei buffer (20 mM 1,4-piperazinediethane sulfonic acid  
20 (pH 7), 3 mM MgCl<sub>2</sub>, 0.5 M hexylene glycol, 10 mM  
orthophenanthroline, 10 mM NaHSO<sub>3</sub>). Triton X-100 is added  
to a final concentration of 1% and the mixture centrifuged  
at 300 g, 4°C for 5 minutes. The nuclear pellet is  
resuspended in nuclei buffer and lysed by the addition of  
25 an equal volume of 30 mM EDTA, 1.5 M NaCl, and 1%  
cetyltrimethylammonium bromide in the presence of  
proteinase K (30  $\mu$ g/ml, Bethesda Research Laboratories)  
for 30 minutes at 65°C. DNA is precipitated in 2.5 M  
NH<sub>4</sub>(Ac, 50% isopropanol precipitation. Five  $\mu$ g of DNA is  
30 digested with BamHI, electrophoresed through 0.8% agarose,  
and transferred (Southern 1975) onto nylon membrane  
(GeneScreen Plus, New England Nuclear). Hybridization is  
carried out with <sup>32</sup>P-labeled synthetic OCS transcripts and  
nick-translated NPT II fragments from pH575, as a control,  
35 or with the various cellulose synthase probes of the  
invention. Filters are prehybridized overnight (in heat-

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sealed bags) at room temperature in 100 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.8), 20 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 5mM EDTA, 1mM orthophenanthroline, 0.1% SDS, 500 µg/ml heparin sulfate, 10% sodium dextran sulfate, and 50 µg/ml each, of heparin sulfate, herring 5 sperm DNA and yeast RNA (Sigma). A portion of the prehybridization solution is removed from the bag, thoroughly mixed with the probe, and then returned to the bag containing the filter. The filter is hybridized for 6-12 h at 65°C and washed (20 MM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM EDTA and 0.1% SDS) at 65°C for 1 h with five buffer 10 changes. Autoradiography is performed on Kodak XAR-5 film with an intensifier screen at -70°C.

Plants whose tissue extracts indicate the presence of 15 cellulose as measured above are selected and tested for enhanced cellulose fiber production. Where applicable, those plants demonstrating improved characteristics will be crossed in order to produce transgenic seed. Any 20 number of indicia may be important to improvement of the plant species thus transformed.

Enhanced or altered cellulose production as a result 25 of the recombinant DNA experiments is deduced by any one or any combination of any of several tests in which the control (non-transformed) and experimental (transformed) plants are compared:

(a) The amount of total cellulose per ovule or per 30 fiber is compared, on a dry weight basis, either before or after alkaline extraction to remove non-cellulosic substances from the cell wall;

(b) The amount of cellulose per fiber as based on a 35 stereology analysis (Zeiss IBAS Image Analysis and AImage Processing System and Image Analysis manual)

of cotton fiber cell wall thickness and fiber length or cotton fiber volume is compared;

5 (c) The polymorph of cellulose present is compared, as determined by x-ray diffraction of untreated

10 cellulose (see procedures described by Alfred D. French in "Cellulose Chemistry and its Applications", Ed. T.P. Nevell and S.H. Zeronian (1985), John Wiley and Sons, New York, Chapter 3, pp 84-111); Also, "Electron Diffraction: An Introduction for Biologists", by D.L. Misell and E.B. Brown, Vol. 12, Practical Methods In Electron Microscopy, Ed. A.M. Glauert (1987), Elsevier, New York, p. 287). Such 15 polymorphs in addition to the native cellulose I might include but may not be limited to cellulose II, cellulose III or cellulose IV (note: only cellulose I is expected to be synthesized in vivo in the control plants);

20 (d) Changes in the average molecular weight of the cellulose are compared, using procedures known to those of skill in the art such as those described by S.B. Ross-Murphy in "Cellulose Chemistry and Its Application", Ed. T. P. Nevell and S.H. Zeronian 25 (1985), John Wiley and Sons, New York, Chapter 8, pp. 202-222;

30 (e) X-ray or electron diffraction patterns of cellulose are compared as described in (c) above to determine changes in the percent crystallinity of the cellulose;

35 (f) Changes in total plant size or changes in specific organ size such as cotton boll size, ovule size, leaf size, stem size, root size, and the like

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as reflected by average cell size within the various tissues and organs of the plant are compared;

5 (g) Ultrastructural morphology of the cellulose, either in the form of microfibrils, elementary fibrils, sub-fibrils, and the like, are compared using transmission electron microscopy and the typical processing known as "negative staining" or standard heavy metal replication techniques whereby 10 the cellulose is made visible for analysis and measurement are carried out [Typical procedures are those found in *Experimental High-Resolution Electron Microscopy* (second edition) by John C.H. Spence, p. 427, Oxford University Press, New York, 1988];

15 (h) Ultrastructural morphology of the cellulose synthesizing complexes is compared, using the freeze-etch/freeze fracture method, as exemplified in "Replica, Shadowing and Freeze-Etching Techniques" by 20 J.H.M. Willison and A.J. Rowe, Vol. 8 of *Practical Methods In Electron Microscopy* Ed. A.M. Glauert, North-Holland Publishing Co., New York (1980) p. 301. See also, "Cellulose Biogenesis and a Decade of 25 Progress: A Personal Perspective", by R. Malcolm Brown, Jr. (1989) pp. 639-657, *In Cellulose and Wood-Chemistry and Technology*, Ed. C. Schuerch, John Wiley and Sons, New York, p. 1638;

30 (i) Uptake of cellulose direct dyes and/or fluorescent brightening agents is compared in which case, the patterns of the controls and experimental plants are differentiated, suggesting changes or alterations in cellulose crystallinity, orientation, etc. See Haigler et al. (1980) "Calcofluor White ST 35 Alters the In vivo Assembly of Cellulose Microfibrils", *Science*, 210:903-906; and



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per gram of tissue, greater than 50 kb in average length, and remarkably uniform from sample to sample.

5        Several modifications have been applied to the method described herein in order to extend the application of miniprep procedures to other plant species and it is anticipated that one may use similar alterations in order to fit the technique to specific species. The selection of a particular protocol depends to a large degree on the 10 plant species used. However, the procedure reported here is selected to be suitable for most situations.

#### Procedure

15        Approximately 1 gm of leaf tissue is quick frozen in liquid nitrogen and ground to a fine powder in a 3" mortar and pestle. The resulting powder is transferred with liquid nitrogen into a 30 ml Oak Ridge tube. It is imperative not to let the tissue thaw once frozen until 20 the buffer is added and not to cap the tubes while nitrogen is evaporating.

25        Next, 15 ml of Extraction Buffer (EB: 100 mM Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl, 10 mM mercaptoethanol) is added. For maximum DNA yields, the cells are further broken by grinding the mixture at a low setting (about 3) with a Polytron (Brinkmann Instruments, Inc.). This step is optional and is tested for its efficacy prior to routine use on a specific tissue source.

30        Approximately 1.0 ml of 20% SDS is added to the tubes and is mixed thoroughly by vigorous shaking. The tubes are then incubated at 65°C for 10 minutes. A volume of 5.0 ml of 5 M potassium acetate is then added to the tubes 35 and the tubes are shaken vigorously and incubated 0°C for 20 minutes.

This procedure is expected to remove most proteins and polysaccharides as a complex with the insoluble potassium dodecyl sulfate precipitate. Precipitation from 0.3 M sodium acetate using relatively small amounts of 5 isopropanol (about 0.6 volumes) has been reported to [REDACTED]

[REDACTED] (Marmur, 1961). The sodium acetate also yields a tight fibrous precipitate that is easily washed and dried. The DNA dissolves readily if allowed to rehydrate at 4°C for 1 10 hour followed by light vortexing.

The tubes are then centrifuged at 25,000 x g for 20 minutes. The supernatant is poured through a Miracloth filter (Calbiochem) into a clean 30 ml tube containing 10 15 ml isopropanol. The resulting solution is mixed and incubated at -20°C for 30 minutes.

The DNA is pelleted at 20,000 x g for 15 minutes. The supernatant is gently decanted and the resulting 20 pellets are lightly dried by inverting the tubes on paper towels for 10 minutes. DNA pellets are redissolved with 0.7 ml of 50 mM Tris, 10 mM EDTA, pH 8 and the solution is transferred to an Eppendorf tube. These tubes are then 25 centrifuged in a microfuge for 10 minutes to remove insoluble debris.

The supernatant is then transferred to a new Eppendorf tube containing 75 µl 3M sodium acetate and 500 µl isopropanol, mixed well and the clot of DNA is 30 pelleted in a microfuge for 30 seconds. The pellet is then washed with 80% ethanol, dried, and redissolved in 100 µl of 10 mM Tris, 1 mM EDTA, pH 8.

Minipreps can be stored for several months without 35 evidence of degradation and can be restricted with a variety of restriction enzymes and ligated without further

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purification. It has been found previously that 10.0  $\mu$ l of miniprep DNA is sufficient for a single 8 mm lane in an agarose gel which is to be used for filter hybridization with single-copy probes. Heat-treated RNAase must be  
5 added to the restriction reaction to digest contaminating RNA in each prep. Hence, a typical reaction contains the following:

	Miniprep DNA	10.0 $\mu$ l
10	10X Restriction Buffer	3.0 $\mu$ l
	0.5 mg/ml RNAase	2.0 $\mu$ l
	<u>EcoRI</u>	8 units
	dH2O to 30 $\mu$ l.	

15 Digestion is usually complete after 3 hours at 37°C. Occasionally, minipreps are difficult to digest with certain enzymes. This problem can be overcome by adding 5.0  $\mu$ l of 0.1 M spermidine to the entire miniprep before digestion (Focus 1982).

20

\*\*\*\*\*

Studies by the present inventors and others support the conclusion that many cellulose synthases will be similar enough in primary structure to allow detection by the technique outlined above or by similar techniques.  
25 For instance, based on the similarities observed between the terminal synthesizing complexes found at the growing tip of microfibrils of cellulose and similarities in the cellulose organization, it has been possible to construct a phylogenetic pathway for cellulose biogenesis. Where such similarities are adequate, the methods and  
30 compositions of the present invention allow the detection and isolation of the similar gene and/or the similar polypeptide.

**EXAMPLE VIII: CLONING OF B-1,4 GLUCAN SYNTHASE GENES  
IN CYANOBACTERIAL/E. COLI SHUTTLE VECTORS**

Using the methods and compositions of the present  
5 invention, two recombinant plasmids are constructed for  
transfer of a  $\beta$ -1,4 glucan synthase such as the [REDACTED]  
[REDACTED]. First, a DNA  
fragment from the plasmid pIS532, carrying the intact  
cellulose synthase gene along with the promoter sequences  
10 from *A. xylinum*, is cloned in the shuttle vector pUC303  
(Kuhlemeier et al. 1983). Expression of the cellulose  
synthase gene is under the control of the *A. xylinum*  
promoter in this recombinant plasmid. Second, the coding  
region of cellulose synthase is cloned in the shuttle  
15 vector pPL191 (Friedberg 1988) where it is under the  
control of the operator promoter regions  $O_L P_L$ ,  $O_R P_R$ , and  
the temperature-sensitive repressor gene *cI857* of  
bacteriophage lambda. These regulatory sequences are used  
for monitoring activity of reporter genes in a  
20 cyanobacterium background (Friedberg and Seijffers, 1986).

**Transformation of *Anacystis Nidulans* R2-SPc**

*Anacystis nidulans* R2 is the strain of choice for  
25 molecular genetic studies in cyanobacteria for which a  
complete system of gene cloning and analysis has been  
developed. This strain carries two plasmids of molecular  
weights 8 Kb and 50 Kb. The 8 Kb plasmid (pUH24) is used  
for the construction of most shuttle vectors, including  
30 pUC303 and pPL191 mentioned above. In order to avoid  
recombination between the vector and the pUH24 present in  
*A. nidulans* R2, use is made of the pUH24-cured strain, R2-  
SPc, for transformation with these vectors.

35 *A. nidulans* R2-SPc is grown in BG-11 medium (Rippka  
et al. 1979) at 30°C with continuous illumination of 250-

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800 foot candles of fluorescent or mixed tungsten-fluorescent light. Cells from a 30 ml culture are pelleted by centrifugation at 5000 rpm in a Sorvall SS34 rotor for 5 minutes. The pellet is resuspended in 15 ml of 10mM NaCl and the centrifugation repeated. The pellet is finally resuspended in 3 ml of BG-11 medium ( $5 \times 10^8$  to  $1 \times 10^9$  cells/ml). 300  $\mu$ l aliquot of cells are transferred to sterile 1.5 ml Eppendorf tubes and 10 ng to 1  $\mu$ g of recombinant plasmid DNA is added. The tubes are then incubated in the dark at 30°C for 4-16 hours with gentle agitation, following which 100-150  $\mu$ l aliquots of each transformation reaction are spread on 100 mm plates containing 40 ml of BG-11 agar. The plates are incubated under standard illuminated growth conditions for 4-6 hours prior to the addition of the required selective agent. For pUC303 derived plasmid, either chloramphenicol (7.5  $\mu$ l/ml) or streptomycin (10  $\mu$ g/ml) is used. Where transformation has been done with pPL191 derived plasmid, kanamycin (25  $\mu$ g/ml) is used for selecting the transformed cells. The antibiotics are added by lifting the agar slab with an alcohol-flamed spatula and dispensing 400  $\mu$ l of a 100x concentrated stock underneath. Transformed colonies typically appear within 4-7 days of incubation in the light at 30°. The transformants are purified on BG-11 agar plates containing the suitable antibiotic, and analyzed for their plasmid content.

Expression of *A. Xylinum* Cellulose Synthase Gene in *A. Nidulans*

The expression of cellulose synthase gene from its own promoter (cloned in pUC303) and the bacteriophage lambda promoter (cloned in pPL191) are monitored at the level of RNA synthesis, cellulose synthase polypeptide synthesis, cellulose synthase activity and cellulose biosynthesis. RNA from the transformed cells is isolated

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essentially as described by Golden et al. (1987) and analyzed by Northern analysis. The synthesis of the cellulose synthase polypeptide in these transformants is determined by western blot analysis using polyclonal antibodies raised against the *A. xylinum* cellulose synthase. Golden

10 used as the substrate according to standard procedures herein. Whether the transformed cells synthesize cellulose is determined by isolating the alkali-insoluble material from these cells and analyzing it by X-ray diffraction.

**EXAMPLE IX: CELLULOSE SYNTHASE ENZYME COMPLEXES PRODUCE CELLULOSE I PRODUCT**

15 Standard conditions for isolation and preparation of  
membrane fractions from Acetobacter were used (see Lin and  
Brown, 1989). Membrane fractions from disintegrated cells  
(using the French Press) were treated with trypsin as  
20 listed in the protocol of Lin and Brown, 1989. This was  
called the "MF" fraction. Unlike Lin and Brown, 1989, the  
MF fraction was treated with 0.6% Triton to solubilize the  
membrane proteins. To this mix were added the ingredients  
for the in vitro reaction mixture which included  $\text{Ca}^{++}$ ,  
25  $\text{Mg}^{++}$ , UDP-glc, and purified cellulose synthase activator,  
cyclic di-GMP. Unlike the usual procedures, no membranes  
were separated by differential centrifugation, and the  
entire reaction mixture with membranes was allowed to  
synthesize cellulose in vitro.

30 The cellulose product (as shown in Fig. 15) is in the form of extended subfibrils with uniform dimensions of approximately  $2.0 \times 2.0$  nm x and at least 200nm in length. This provides clear proof that the in vitro synthesized cellulose polymorph is cellulose I, the same type and morphology of that which is assembled in vivo. Cellulose  
35

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I polymorphs can only be synthesized in vitro when the UDP-glc substrate concentration is sufficiently high (in this case, 20.0mM). If this substrate is used in a lower concentration range such as 1.9mM, only cellulose II is 5 synthesized as shown in Lin and Brown, 1989 (also see Bureau and Brown 1987). The rodlets of cellulose II are only 35 nm long and have variable widths, up to 5.4nm.

By sufficiently saturating all of the available UDP-10 glc binding sites of the isolated enzyme complex, multiple parallel glucan chains are polymerized in sufficient close proximity to crystallize into the metastable cellulose I polymorph. If these sites are undersaturated, the glucan chains may have the capacity to fold and collapse into the 15 more stable cellulose II polymorph. Cellulose I can be converted to cellulose II (and not vice versa) by the process of mercerization or treatment with concentrated alkali which dissolves the crystalline cellulose, then as the alkali is removed, precipitates to form the more 20 stable cellulose II polymorph.

It should be noted that the 2.0 x 2.0nm subfibril is itself too small to be highly crystalline; however, if 25 multiple subfibrils coalesce as they do in vivo, a stable, highly crystalline cellulose I polymorph is formed. Thus, a necessary prerequisite for cellulose I formation in vivo or in vitro is the formation of a stable, extended subfibril of relatively constant dimensions, and the enzyme complex must be fully saturated with substrate to effect 30 this synthesis.

The present invention has been described in terms of particular embodiments found or proposed by the present inventors to comprise preferred modes for the practice of 35 the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure,

numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence.

5

modifications, changes can be made in the protein structure without affecting in kind or amount of the biological action. All such modifications are intended to be included within the scope of the appended claims.

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CLAIMS:

1. A recombinant vector incorporating a DNA segment having a sequence encoding a  $\beta$ -1,4 glucan synthase 5 polypeptide.

2. The vector of claim 1, where the sequence encodes a polypeptide which includes the amino acids 1 to 10 723 of Figure 1.

3. The vector of claim 2 where the sequence encodes a polypeptide consisting essentially of amino acids 1 to 15 723 of Figure 1.

4. The vector of claim 1, where the sequence encodes a polypeptide which includes the amino acids 1 to 20 809 of Figure 13.

5. The vector of claim 4 where the sequence encodes a polypeptide consisting essentially of amino acids 1 to 25 809 of Figure 13.

6. The vector of claim 1, where the  $\beta$ -1,4 glucan synthase polypeptide encoding sequence is positioned adjacent to and under the control of an effective 30 promoter.

7. The vector of claim 6, where the promoter comprises a prokaryotic promoter, the vector being adapted 35 for expression in a prokaryotic host.

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8. The vector of claim 6, where the promoter comprises a eukaryotic promoter, the vector being adapted for expression in an eukaryotic host, and the vector further includes a polyadenylation signal positioned 3' of the carboxy-terminal amino acid, and within a transcriptional unit of the vector.

9. A DNA segment having a sequence encoding a  $\beta$ -1,4 glucan synthase polypeptide.

10. The DNA segment of claim 9, where the sequence encodes a polypeptide corresponding to amino acids 1 to 15 723 of Figure 1.

11. The DNA segment of claim 9 where the sequence encodes a polypeptide consisting essentially of amino acids 1 to 723 of Figure 1.

12. The DNA segment of claim 9, where the sequence encodes a polypeptide corresponding to amino acids 1 to 25 809 of Figure 13.

13. The DNA segment of claim 9, where the sequence encodes a polypeptide consisting essentially of amino acids 1 to 809 of Figure 13.

14. A DNA segment having a sequence comprising at least a 14 base sequence of the DNA sequence of Figure 1, 35 or its complement, the segment capable of hybridizing to the DNA sequence of Figure 1, or its complement.

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15. The DNA segment of claim 14, where the sequence comprises at least an 18 base sequence of the DNA sequence of Figure 1.

5

16. A DNA segment having a sequence consisting essentially of at least a 14 base sequence of the DNA sequence of Figure 1.

10

17. A DNA segment having a sequence comprising at least a 14 base sequence of the DNA sequence of Figure 13, or its complement, the segment capable of hybridizing to 15 the DNA sequence of Figure 13, or its complement.

18. The DNA segment of claim 17, where the sequence comprises at least an 18 base sequence of the DNA sequence 20 of Figure 13.

19. A DNA segment having a sequence consisting essentially of at least a 14 base sequence of the DNA 25 sequence of Figure 13.

20. A recombinant vector incorporating a DNA segment as defined by any one of claims 9 through 19.

30

21. A  $\beta$ -1,4 glucan synthase polypeptide.

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22. The  $\beta$ -1,4 glucan synthase polypeptide of claim 21 further defined as including amino acids 1 to 723 of Figure 1.

5

23. The  $\beta$ -1,4 glucan synthase polypeptide of claim 21 further defined as a recombinant polypeptide.

10 24. The  $\beta$ -1,4 glucan synthase polypeptide of claim 21 further defined as including amino acids 1 to 809 of Figure 13.

15 25. The  $\beta$ -1,4 glucan synthase polypeptide of claim 21 further defined as a recombinant polypeptide.

20 26. An antibody immunologically reactive with any one of the polypeptides of claim 21 through 24.

25 27. The antibody of claim 25, further defined as a monoclonal antibody.

28.

28. A method of purifying a  $\beta$ -1,4 glucan synthase polypeptide, comprising:

30 (a) culturing cells capable of producing  $\beta$ -1,4 glucan polymers so that  $\beta$ -1,4 glucan synthase is expressed;

35 (b) partially purifying the  $\beta$ -1,4 glucan synthase from the cells by solubilizing the cellular membranes;

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(c) identifying an active subunit of the  $\beta$ -1,4 glucan synthase; and

5 (d) further purifying the active subunit of the  $\beta$ -1,4 glucan synthase.

29. The method of claim 28 where the cells capable of  $\beta$ -1,4 glucan polymer synthesis are cells of cotton.

10

30. The method of claim 28 where further purifying the active subunit of the  $\beta$ -1,4 glucan synthase comprises purifying the  $\beta$ -1,4 glucan synthase by product entrapment.

15

31. The method of claim 28 where identifying the active subunit of  $\beta$ -1,4 glucan synthase further comprises admixing a  $\beta$ -1,4 glucan synthase activator with the 20 partially purified  $\beta$ -1,4 glucan synthase.

32. The method of claim 28 where identifying the active subunit of  $\beta$ -1,4 glucan synthase further comprises 25 photoincorporating onto the partially purified  $\beta$ -1,4 glucan synthase a radioactively labelled azidonucleotide analog capable of specifically interacting with the nucleotide binding site of  $\beta$ -1,4 glucan synthase.

30

33. The method of claim 28 where further purifying the active subunit of the  $\beta$ -1,4 glucan synthase comprises purifying the  $\beta$ -1,4 glucan synthase by electrophoresing the labeled  $\beta$ -1,4 glucan synthase through a gel matrix.

35

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34. The method of claim 28 where the cells capable of  $\beta$ -1,4 glucan polymer synthesis are cells of a strain of Acetobacter xylinum, cells of an Agrobacterium species, cells of a Rhizobium species or cells of a Sarcina species.

5

35. The method of claim 28 where partially purifying  $\beta$ -1,4 glucan cellulose synthase from the cells by solubilizing the cellular membranes is accomplished using digitonin.

10

36. The method of claim 28 where the  $\beta$ -1,4 glucan synthase activator is bis-(3',5')-cyclic diguanylic.

15

37. The method of claim 28 where the  $\beta$ -1,4 glucan synthase activator is c-di-GMP.

20

38. The method of claim 28 where the  $\beta$ -1,4 glucan synthase activator is contained in a crude extract of A. xylinum cells.

25

39. A recombinant host cell which incorporates a DNA sequence in accordance with claim 9.

30

40. A recombinant host cell which incorporates DNA sequences encoding more than one glucan synthase polypeptide.

35

41. The recombinant host cell of claim 39 further defined as a eukaryotic host cell.

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42. The recombinant host cell of claim 39 further defined as a prokaryotic host cell.

5

43. The prokaryotic host cell of claim 42 further defined as a cyanobacterium.

10

44. The recombinant host cells of claims 39 through 43 where the DNA segment encoding a  $\beta$ -1,4 glucan synthase polypeptide is under the transcriptional control of regulatory signals functional in the recombinant host cell which regulatory signals appropriately control the expression of the glucan synthase polypeptide in a manner to allow all necessary transcriptional and post-transcriptional modification.

20

45. The recombinant host cell of claim 40 where the host cell is a plant entity consisting essentially of a plant cell, seed, or plant having a DNA segment, as a result of the in vitro introduction of the DNA segment into a plant cell, the DNA segment encoding a  $\beta$ -1,4 glucan synthase polypeptide, the DNA segment being under the transcriptional control of regulatory signals functional in the plant entity, which regulatory signals appropriately control the expression of the  $\beta$ -1,4 glucan synthase polypeptide in a manner to allow all necessary transcriptional and post-transcriptional modification.

25

46. The recombinant plant cell, seed or plant of claim 43 which comprises a species of the genus Gossypium.

30

35

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47. The recombinant plant cell, seed or plant of claim 43 which comprises an algae.

5 48. The gene of claim 43 which comprises a gene which is heterologous to the host cell. [REDACTED]  
[REDACTED] gene is introduced.

10 49. A method of producing a  $\beta$ -1,4 glucan synthase polypeptide, comprising:

15 (a) producing a recombinant host cell according to any of claims 38 through 46, such cell being capable of expressing the polypeptide;

20 (b) culturing the host cell under conditions appropriate for expressing the polypeptide; and

(c) recovering the polypeptide.

25 50. A method of producing a  $\beta$ -1,4 glucan polymer, comprising:

30 (a) producing a recombinant host cell according to any of claims 38 through 46, such cell being capable of producing the  $\beta$ -1,4 glucan polymer;

35 (b) culturing the host cell under conditions appropriate for producing the  $\beta$ -1,4 glucan polymer; and

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(c) recovering the  $\beta$ -1,4 glucan polymer.

5 51. The method of claim 50 where the  $\beta$ -1,4 glucan polymer is further defined as cellulose.

10 52. The method of claim 50 where the  $\beta$ -1,4 glucan polymer is a mixed  $\beta$ -1,4 glucan polymer.

15 53. A method of producing a  $\beta$ -1,4 glucan polymer, said polymer further comprising a predetermined amount of a given  $\beta$ -1,4 glucan polymorph, comprising:

20 (a) selecting a cell capable of producing  $\beta$ -1,4 glucan polymer;

(b) culturing the cell under conditions appropriate for producing the  $\beta$ -1,4 glucan polymer;

25 (c) obtaining a cell-free extract of the cell;

(d) providing a at a selected concentration substrate to the cell extract designed to cause the cell extract to form the selected  $\beta$ -1,4 glucan polymorph; and

30 (e) recovering the selected  $\beta$ -1,4 glucan polymer.

35 54. The method of claim 53 where the polymorph is cellulose I.

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55. The method of claim 53 where the substrate is selected from the group of nucleotide sugars consisting of UDP-xylose, UDP-mannose, ADP-glucose, GDP-glucose, and UDP-glucose.

5

56. The method of claim 53 wherein the concentration of substrate is sufficiently high to  
saturate approximately all available substrate binding  
sites on any  $\beta$ -1,4 glucan synthetic enzyme contained  
within the cell-free extract to cause thereby the  
formation of extended sub-fibrils of the selected  $\beta$ -1,4  
glucan polymer which sub-fibrils are of an approximately  
uniform width.

**FIG.1A**

CGCTTCCGC ACATCAGCAT CGTCCATAAG TGAGCGTCCG GTGACAGGGT GTTGCCGATG  
60

AATGGAGTCC TGTTCGGAAG CACGCCCTCAG CCGGGGACG TCCGGCTCCC GGGGATCTGA  
120

GCTTTTCTT TCAGGGATG CGCGACACCC GTGATGAAA GAAGTTCCTT CCCGTGGCGT  
180

CCACGGATC AGTTCGTTCTT AATGTTCCA GGATGACCAG CATGACCAAG ACAGACACGA  
240

ATTCTCTCA GGCTTCTCGT CCCGGCAGCC CCGTCGGCTC GCCTGATGGG TCGCCCCACAA  
300

TGGCCGAAGT GTTCATGACG CTGGTGGTC GTGCCGACGGA ACTCCTCAGC CCCCGTCCTT  
360

CGCTGGGA GGCCTGTTG CGTCGTCGTG AAAACGAAGA AGAATCCTAA GGCCCTATAT  
420

TCAAGGGGACCC GCCTGTCCTT GTGTCCTGCC TGTTGAAATG GGGGGGGCAG GGGCAGACAC  
480

**FIG.1B**

GCTGCACTGG CTCGGCGATC TATTCCGTCT TTGGCCTGGG TGATGTTTCG  
540 SCAGGCCAGG

CGGGCCCCGCT TTGCCCCGAT GTGACCCGAG ATGGCTGGGG TCAGGGTTTT  
600 TTTGACGTAAG

TTTTTCTGTT TTACAGCATT TCGGACGAGT TGTCTT ATG CCA GAG GTT  
1 TGG  
Met Pro Gly Val Arg

TCG TCA ACG CAG TCA GAG TCA GGA ATG CCA CAG TGG ATG GG  
Ser Ser Thr Gln Ser Glu Ser Gly Met Ser Gln Trp Met Gln  
10 15 20  
AAA LYS

ATT CTT TCC ATT CGC GGT GCT GGG CTG ACT ATT GGT GTT TTG  
Ile Leu Ser Ile Arg Gly Ala Gly Leu Thr Ile Gly Val Phe  
25 30 35  
GGC GLY

CTG TGT GCG CTG ATT GCG GCT ACG TCC GTG ACC CTG CCG CCA GAA  
Leu Cys Ala Leu Ile Ala Ala Thr Ser Val Thr Leu Pro Pro  
40 45 50  
785 SLU

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**SUBSTITUTE SHEET**

**FIG.1C**

CAG CAG TTG ATT GTG GCA TTT GTA TGT GTC GTG ATC TTT TTT ATT  
 Gln Gln Leu Ile Val Ala Phe Val Cys Val Val Ile Phe Phe Ile  
 55 60 65

GTC GGT CAT AAG CCC AGC CGT CGG TCC CAG ATT TTC CTT GAA GTG 875  
 Val Gly His Lys Pro Ser Arg Arg Ser Gln Ile Phe Leu Glu Val  
 70 75 80

3 / 24  
 CTG TCA GGG CTG GTT TCG CTG CGC TAT CTG ACA TGG CGC CTG ACG 920  
 Leu Ser Gly Leu Val Ser Leu Arg Tyr Leu Thr Trp Arg Leu Thr  
 85 90 95

GAA ACG CTT TCA TTC GAT ACA TTG CAG GGT CTG CTT GGG ACA 965  
 Glu Thr Leu Ser Phe Asp Thr Trp Leu Gln Gly Leu Leu Gly Thr  
 100 105 110

ATG CTT CTG GTG GCG GAA CTT TAC GCC CTG ATG CTG TTC CTC CTC 1010  
 Met Leu Leu Val Ala Glu Leu Tyr Ala Leu Met Met Leu Phe Leu  
 115 120 125

**FIG.11**

AGC	TAT	TTC	CAG	ACG	ATC	GGC	CCA	TTG	CAT	CGT	GGC	CCT	CT	CCG	1055
Ser	Tyr	Phe	Gln	Thr	Ile	Ala	Pro	Leu	His	Arg	Ala	Pro	Le	Pro	
															140
CTG	CCG	CCG	AAC	CCT	GAC	GAA	TGG	CCC	ACG	GTC	GAT	ATC	TT	GTC	1100
Leu	Pro	Pro	Asn	Pro	Asp	Glu	Trp	Pro	Thr	Val	Asp	Ile	Ph	Val	
															155
CCG	ACC	TAC	AAC	GAA	CTG	AGC	ATT	GTC	CCC	CTG	ACG	GT	CTG	1145	
Pro	Thr	Tyr	Tyr	Asn	Glu	Glu	Leu	Ser	Ile	Val	Arg	Leu	Thr	Leu	
															160
GGA	TCA	CTG	GGG	ATT	GAC	TGG	CCA	CCG	GAA	AAG	GTG	CGG	GT	CAT	1190
Gly	Ser	Leu	Gly	Ile	Asp	Trp	Pro	Pro	Pro	Glu	Lys	Val	Arg	Val	
															175
ATC	CTT	GAT	GAC	GGT	CGT	CCT	GAA	TTT	GCC	GCC	TTT	GCC	GCT	1235	
Ile	Leu	Asp	Asp	Gly	Arg	Arg	Pro	Glu	Phe	Ala	Ala	Phe	Ala		
															195
															200

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**FIG.13C****SUBSTITUTE SHEET**

CTCGACGTACCGCCGTGGACCGTGGTCAAGTTCAGTTGAACTTCTACTTTGACCGCG	4380
L D V P P W T V F G Q L N F Y F D A	507
ATGCCCTTGGCGCGGAATCTGTCAAAGTGGCGGGAAACATGGCGTCCATCTTGGG	4440
M P L A R G I C Q S G A A N N A F H L G	527
CTTGACCCGGATTCCACCATGGATTTCCTCCGTGGCCCATCACATGCCAGATGCCAAC	4500
L D P S T I D F S R A H H I A Q M P N	547
CTTGCTATATGGCGACGGTGGTTTCTTACCACTATGCCGATCTGTCGCAGACG	4560
L A Y M A T V G F P F T T Y A D L S Q T	567
GCGGTTGTTCTGGCTGAACACCCGAATGCCGGACTGTGGCGCTATCTGACCTGATG	4620
A V V L P E H P N A A T V G A Y L D L M	587
<hr/>	
GGGTTCAATGGGGGGGGGACGTGGTATCCGGTTGGTGGACATTGTGTGGCCGAT	4680
G F M G A A T W Y P V A G V D I V S A D	607
<hr/>	
CATGTCAAGTGAACGGTGGACCGTAACCTGGTGGTGGATTTGACGGCTGGCGACAAAGTGGC	4740
H V S D V A D R N L L V I S T L A T S G	627
GAGATCGGGCGCTGGCTGTCAACGGTCACTCCTACGAAGTGGGATGGTCATTGGCACG	4800
E I A P L L S R S Y E V A D G H L R T	647
GTTGGCATGGGTCCGGCTGGATAACGGGATCAAAGGGATCCGACGATCCGCTGACGGCT	4860
V S H A S A L D N A I K A V D D P L T A	667

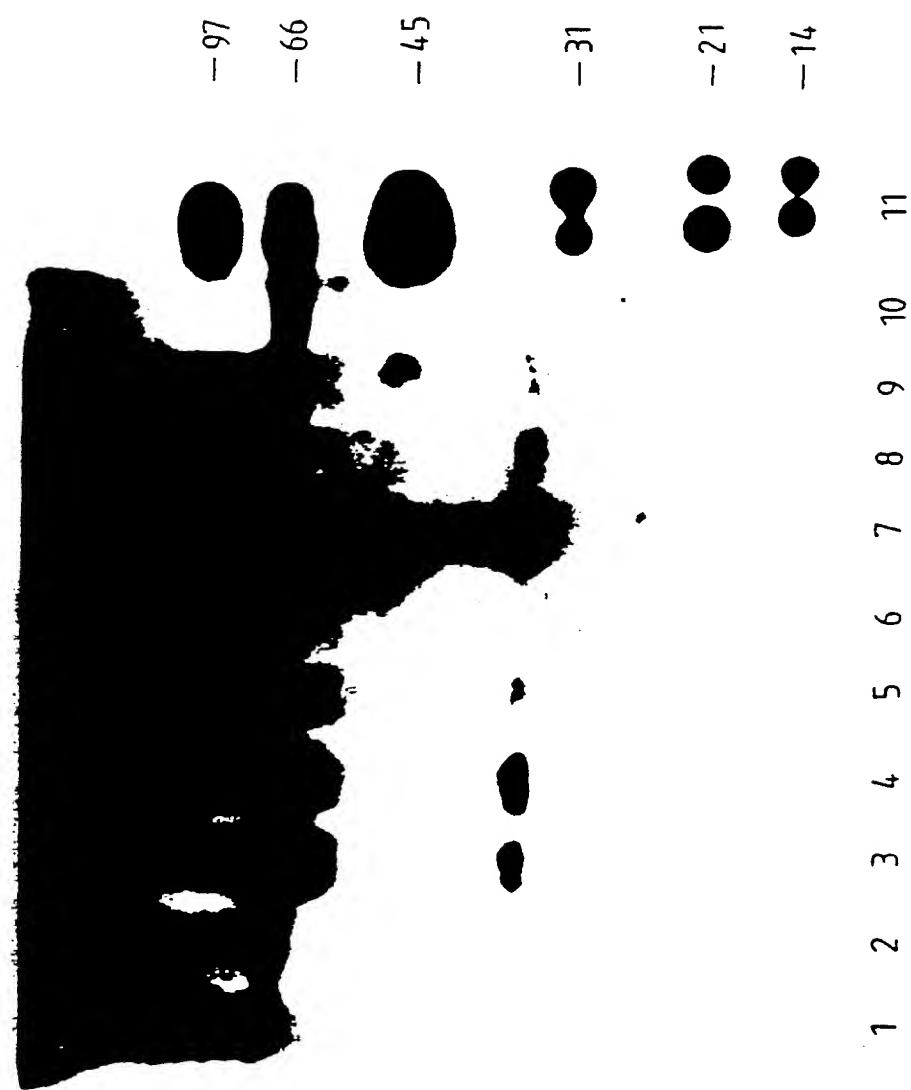
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**FIG.13 D**

TTCCGGGACCGGTGACAGCAAGCCGAGGATGTCGATACCCCCCTGACGGGTGGTCGGC  
 F R D S K P Q D V D T P L T G G V G 4920  
 GCGATGATCGAGGGGAATCCCCCTGACGGGGCGCACCGTTCTGGCCCTGCTGTCG  
 A M I E A E S P L T A G R T V L A L S 687 4980  
 TCTGACGGTGGGGCTGAACAACTCCCTCAGATGTTGGGGAGGCAAGAACGAGGGCG  
 S D G A G L N N L L Q M L G E R K K Q A 707 5040  
 AATATCCAGGGGACCTGGTTGGCCATGGCGAGGGACCTGTTCTTGTACCGCACTTCG  
 N I Q G D L V A H G E D L S S Y R T S 727 5100  
 CCGGTCTATAAGATCGGCACCCCTGGCCTCTGGCTGTGGGGACTGGTATATGCCAAC  
 P V Y T I G T L P L W P D W Y M H N 747 5160  
 AGACCCGGTTGGTGTGGCTGCTGGCTGTGGGATGTATTCTGATCGTCAGTGTCTT  
 R P V R V L L V G L L G C I L I V S V L 767 5220  
 GCACGGGCTTGGCGGGCATGGGACCCGGCTTCAAGCAGGCTTGAGGATGAGAGGGCG  
 A R A L A R H A T R R F K Q L E D E R R 807 5340  
 AAGTCGGTGGCCATAAACGATATGCTTCGTCCTGGGGCTTCCTCGCAACGACCT  
 K S STOP 809 5363  
 GCGTCCGGTCTGCTGCAAG

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FIG. 14



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FIG.15



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ISOCID: WO\_9113988A1\_>

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/01726

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 C12N15/54 ; A01H5/00 ; C12P19/04 ; C12Q1/68

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
Int.C1. 5	C12N ; C12R

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

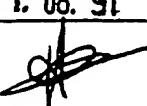
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	LIN, F.C. & BROWN, R.M.: "Cellulose and wood - chemistry and technology" 1989, Schuerch, C., Ed., Wiley, New-York see pages 473 - 492	21-22, 24, 28, 30-31, 33-38
Y	(cited in the application) ---	1-20, 39, 42, 44
Y	US,A,4788135 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD, JR UNIVERSITY) 29 November 1988 see the whole document ---	1-20, 39, 42, 44 -/-

<sup>6</sup> Special categories of cited documents :<sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

<sup>7</sup> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<sup>8</sup> "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step<sup>9</sup> "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.<sup>10</sup> "&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
3 21 JUNE 1991	01.08.91
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer ANDRES S.M. 

III. DOCUMENTS CONSIDERED TO BE RELEVANT		(CONTINUED FROM THE SECOND SHEET)
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	SCIENCE. vol. 230, 15 November 1985, LANCASTER, PA US pages 822 - 825; LIN, F.C. et al.: "Synthesis of fibrils in vitro by a solubilized cellulose synthase from <i>Acetobacter xylinum</i> " see the whole document	21, 31, 34-35, 37-38  49-51, 53-56
Y	(cited in the application) --- JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 264, no. 20, 15 July 1989, BALTIMORE US DRAKE, R.R. et al.: "Synthesis and properties of 5-azido-UDP-glucose " pages 11928 - 11933 see the whole document (cited in the application) ---	32-33  28, 32-33
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 84, October 1987, WASHINGTON US pages 6985 - 6989; BUREAU, T.E. et al.: "In vitro synthesis of cellulose II from a cytoplasmic membrane fraction of <i>Acetobacter xylinum</i> " see the whole document (cited in the application) ---	21, 28, 31, 34-36, 49-51, 55
P, X	PLANT MOLECULAR BIOLOGY. vol. 15, 1990, DORDRECHT NL pages 673 - 683; SAXENA, I.M. et al.: "Cloning and sequencing of the cellulose synthase catalytic subunit gene of <i>Acetobacter xylinum</i> " see the whole document	1-3, 6-7, 9-11, 14, 16
P, X	(cited in the application) --- JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 265, no. 9, 25 March 1990, BALTIMORE US pages 4782 - 4784; LIN, F.C. et al.: "Identification of the uridine 5'-diphosphoglucose (UDP-Glc) binding subunit of cellulose synthase in <i>Acetobacter xylinum</i> using the photoaffinity probe 5-azido-UDP-Glc" see the whole document (cited in the application) ---	20-23, 28, 30-35, 37-39  21, 28, 30-38, 49

-/-

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P, X	<p>WO, A, 9012098 (CETUS CORPORATION) 18 October 1990        see page 15, line 6 - page 19, line 13</p> <p>see page 37, line 15 - page 38</p> <p>see page 43, line 11 - page 46, line 30</p> <p>see page 51, line 25 - page 54, line 25</p> <p>see pages 60 - 66, line 26</p> <p>see page 78, line 13 - page 81, line 13; figure 1</p> <p>---</p>	1, 6-7, 9, 20-21, 25, 28 30-31, 34-35, 37-40, 42-44 49-51, 53, 55

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.

SA 46083

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 21/06/91

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4788135	20-11-89	-----	-----
WO-A-9012098	18-10-90	AU-A- 5437390	05-11-90